



# Dissecting Protein-Protein Interactions that Regulate the Spindle Checkpoint in Budding Yeast

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## **Dissecting Protein-protein Interactions that Regulate the Spindle Checkpoint in Budding Yeast**

### **Abstract**

Errors in segregation of genetic materials are detrimental to all organisms. The budding yeast ensures accurate chromosome segregation by employing a system called the spindle checkpoint. The spindle checkpoint, which consists of proteins such as Mad1, Mad2, Mad3, Bub1, and Bub3, monitors the attachment of microtubules to the chromosomes and prevents cell cycle progression until all chromosomes are properly attached.

To understand how the spindle checkpoint arrests cells in response to attachment errors at the chromosomes, we recruited different checkpoint proteins to an ectopic site on the chromosome by taking advantage of the binding of the lactose repressor (LacI) to the lactose operator (LacO). We found that cells expressing Bub1-LacI arrest in metaphase. The phenotype is in fact caused by dimerization of Bub1 when it is fused to LacI rather than the recruitment of Bub1 to chromosome. The cell cycle arrest by the Bub1 dimer depends on the presence of other checkpoint proteins, suggesting that the dimerization of Bub1 represents an upstream event in the spindle checkpoint pathway.

The results with the Bub1 dimer inspired us to fuse checkpoint proteins to each other to mimic protein interactions that may contribute to checkpoint activation. We showed that fusing Mad2 and Mad3 arrests cells in mitosis and that this arrest is

independent of other checkpoint proteins. We believe that combining Mad2 and Mad3 arrests cells because both proteins can bind weakly to Cdc20, the main target of the spindle checkpoint, and the sum of these two weak bindings creates a hybrid protein that binds tightly to Cdc20. We reasoned that if Mad3's role is to make Mad2 bind tightly, artificially tethering Mad2 directly to Cdc20 should also arrest cells and this arrest should not depend on any other checkpoint components. Our experiments confirmed these predictions, suggesting that Mad3 is required for the stable binding of Mad2 to Cdc20 in vivo, that this binding is sufficient to inhibit APC activity, and that this reaction is the most downstream event in spindle checkpoint activation. The interactions among spindle checkpoint proteins thus play an important role in cell cycle arrest and must be carefully regulated.



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*For my Parents*



## **Chapter One**

### **Introduction**

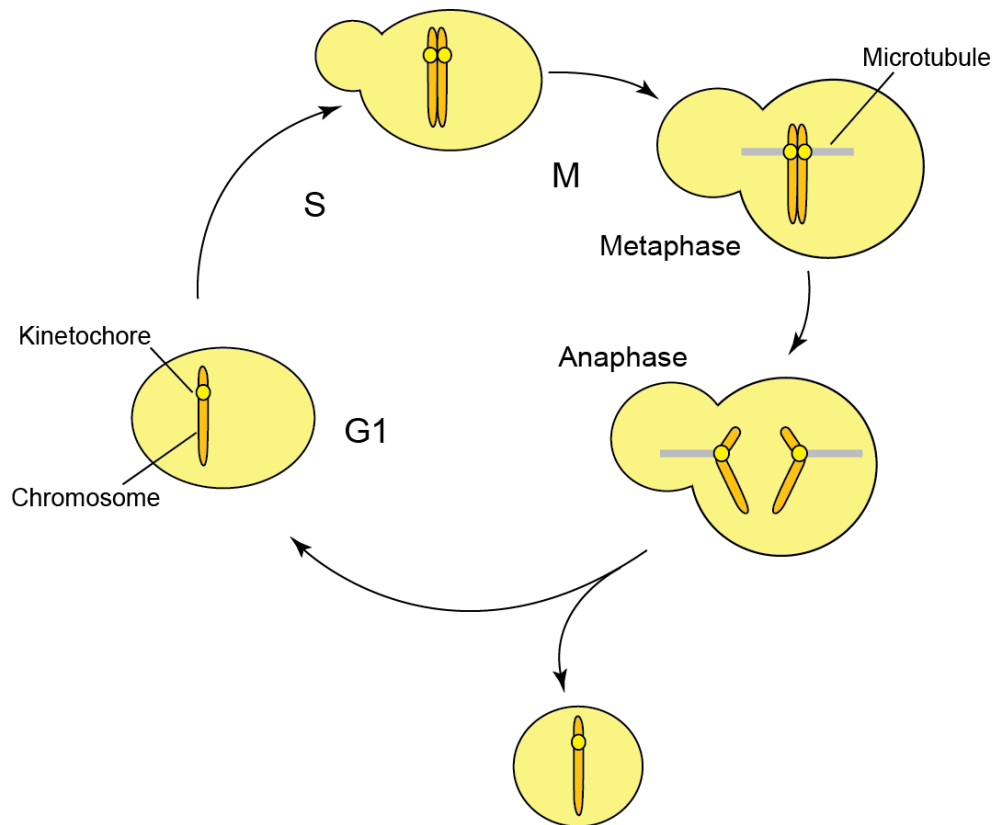
## **Abstract**

A cell has to undertake the complicated tasks of duplicating and segregating its genetic materials in order to proliferate. Eukaryotes have multiple systems to ensure the order and fidelity of these events. Chromosomes are separated during mitosis by microtubules, which attach to chromosomes by binding to a protein complex called the kinetochore. The spindle checkpoint ensures the accuracy of chromosome segregation by monitoring microtubule attachments at the kinetochores. When even a single kinetochore is not properly attached, the spindle checkpoint stops cell cycle progression and provides an opportunity for the cells to correct the attachment errors. The cell cycle arrest is achieved by inhibiting the E3 ubiquitin ligase named the anaphase-promoting complex (APC) and its co-activator Cdc20. How events at the kinetochore lead to inhibition of APC<sup>Cdc20</sup> by the spindle checkpoint components is still not clear. This chapter will summarize what the field currently knows about the spindle checkpoint by focusing on how checkpoint proteins interact with the kinetochore and how the checkpoint ultimately inhibits the activity of APC<sup>Cdc20</sup>.

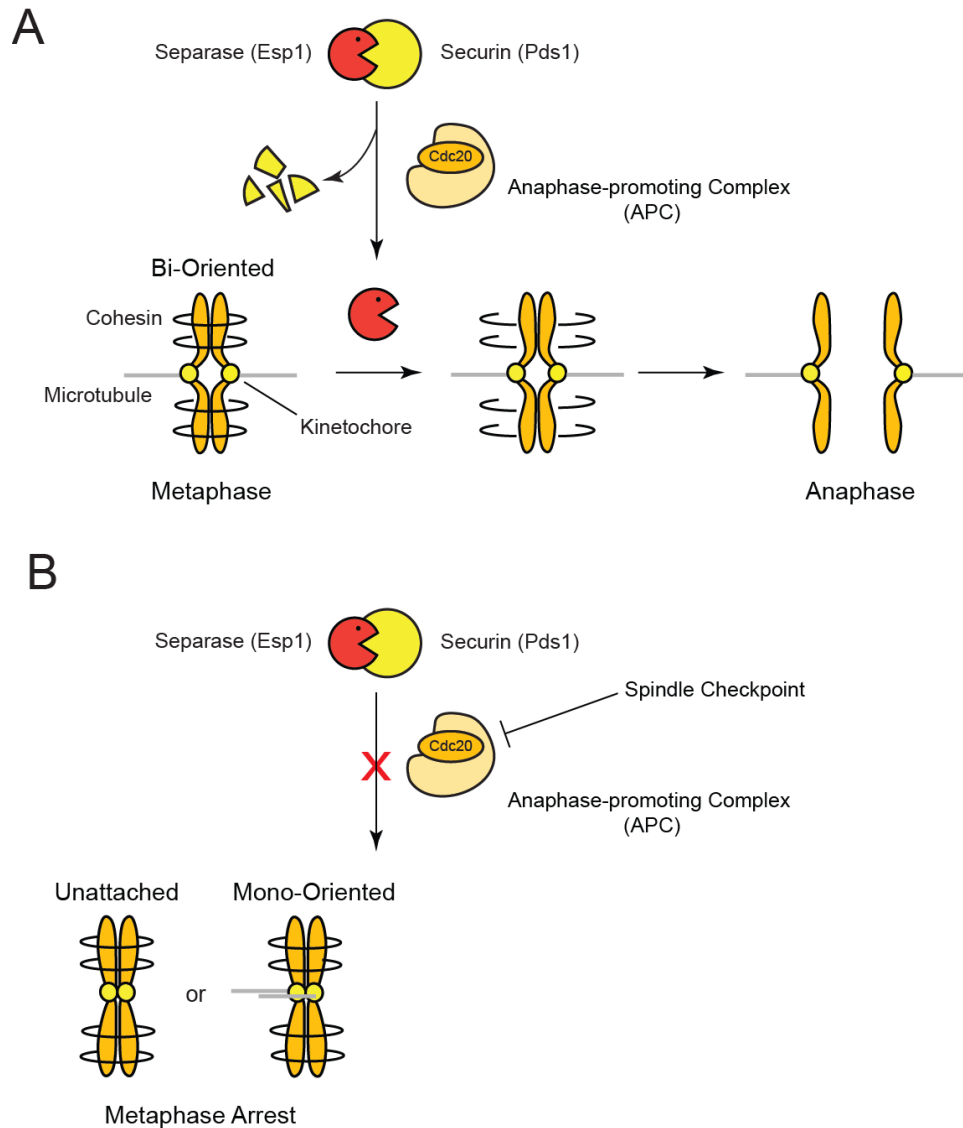
## Introduction

The cell cycle consists of a set of processes a cell has to execute in a controlled and timely manner to successfully form two viable and genetically identical cells (**Figure 1-1**) (reviewed in [1]). One main event during the cell cycle is the duplication and segregation of genetic materials. In the budding yeast, *Saccharomyces cerevisiae*, the 16 chromosomes replicate exactly once in synthesis (S) phase. The two identical copies of sister chromosomes are held together by the protein complex cohesin and are segregated from each other during mitosis, an elaborate process that consists of multiple steps. In metaphase, microtubules line up the duplicated chromosomes in the middle of the cells by attaching to the two sister kinetochores, specialized multi-protein structures that assemble on centromeric DNA and capture microtubules (reviewed in [2]). An E3 ubiquitin ligase known as the anaphase-promoting complex (APC) and its co-activator Cdc20 then catalyze the ubiquitination and destruction of securin (Pds1 in budding yeast), which leads to the release of active separase (Esp1 in budding yeast) (**Figure 1-2A**). Separase cleaves cohesin and destroys the linkage between sister chromosomes, ultimately triggering anaphase and chromosome segregation.

Errors during the duplication and separation of chromosomes are usually detrimental to the cells. The accuracy of these events relies on the presence of multiple checkpoints, which generally work by preventing the cells from entering the next phase of the cell cycle unless the previous step is properly carried out. The spindle checkpoint, first identified in the budding yeast [3, 4], ensures correct chromosome segregation by monitoring the attachment and alignment of chromosomes on the mitotic spindle (reviewed in [5, 6]). When even a single chromosome is not properly attached, the



**Figure 1-1.** The budding yeast cell cycle. In G1 phase, budding yeast cells grow until they reach a size threshold. Cells then start budding and duplicating their chromosomes during the synthesis (S) phase. After that, cells enter mitosis, during which sister chromosomes move along microtubules to congress to the middle of the cell (metaphase) and are then segregated into the mother and daughter cell (anaphase). Cytokinesis and cell separation then occurs, and the two new cells can repeat the cell cycle to continue forming new progeny.



**Figure 1-2.** The control of metaphase-to-anaphase transition. **(A)** After DNA replication, the two sister chromosomes are held together by the protein complex cohesin. In metaphase, microtubules from opposite spindle poles attach to the duplicated chromosomes at the two sister kinetochores. After all the chromosomes are properly attached, an E3 ubiquitin ligase known as the anaphase-promoting complex (APC) and its co-activator Cdc20 catalyze the ubiquitination and destruction of securin (Pds1 in budding yeast), thus releasing active separase (Esp1 in budding yeast). Separase cleaves cohesin and destroys the linkage between sister chromosomes, ultimately triggering anaphase and chromosome segregation. **(B)** When a single chromosome becomes unattached or when its two sister kinetochores are attached to microtubules from the same spindle pole, the spindle checkpoint is activated. The main goal of the checkpoint is to inhibit the activity of APC<sup>Cdc20</sup>. Inhibition of APC<sup>Cdc20</sup> stabilizes securin and keeps separase inactive. The spindle checkpoint hence arrests cells in metaphase until all chromosomes are properly attached.

spindle checkpoint will be activated. The main function of the spindle checkpoint is to inhibit the activity of APC<sup>Cdc20</sup>, thus stabilizing securin and arresting cells before the metaphase-to-anaphase transition (**Figure 1-2B**). The cell cycle arrest allows cells to achieve proper microtubule attachment at the kinetochores before progressing through the cell cycle, thus preventing chromosome missegregation.

The components of the spindle checkpoint have been identified by several clever genetic screens which look for mutants that fail to respond to improper microtubule attachments. The spindle checkpoint components include the mitotic-arrest deficient (Mad) proteins Mad1, Mad2, and Mad3 [3], the budding uninhibited by benzimidazole (Bub) proteins Bub1 and Bub3 [4], Mps1 [7], Aurora B (Ipl1 in yeast) [8], and Sgo1 [9], all of which are highly conserved among eukaryotes. Despite knowing the identity of the main players, we still lack a detailed molecular description of how attachment errors at the kinetochore are converted into signals that inhibit APC<sup>Cdc20</sup>.

Below I will focus on two aspects of the spindle checkpoint that are crucial to our understanding of the system: (1) how the kinetochore interacts with components of the spindle checkpoint; and (2) how the spindle checkpoint proteins coordinate with each other to inhibit APC activity. I will summarize what we currently know about these areas as well as the unanswered questions that motivated my thesis work.

## **The Kinetochore: the Source of Spindle Checkpoint Signals**

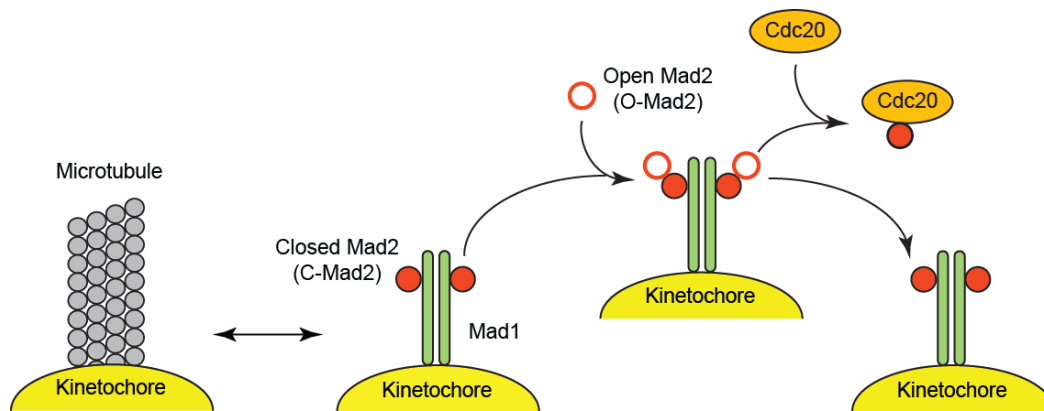
The spindle checkpoint is activated when chromosomes fail to attach to the mitotic spindle properly. Normal chromosome segregation depends on the two sister kinetochores attaching to microtubules from opposite spindle poles during mitosis (bi-orientation). The pulling force from the microtubules is balanced by cohesion between the sister chromatids, generating tension across the kinetochores. Bi-orientation is the only attachment state that supports an even segregation of chromosomes during mitosis. The spindle checkpoint is activated by the absence of microtubule attachment [3, 4, 10] or the lack of tension at the kinetochore (because of sister chromosomes attaching to the same spindle pole) [11-13] and leads to cell cycle arrest (**Figure 1-2B**), thus ensuring that all chromosomes are bi-oriented before the cell enters anaphase.

The kinetochore, with its role in binding microtubules, is thought to be the major site for generating spindle checkpoint signals. Supporting this, budding yeast mutants with compromised kinetochore structures fail to activate the checkpoint even in the presence of microtubule poisons [14, 15], showing that kinetochore components are normally required to initiate the checkpoint pathway. It is important for cells to only arrest when the chromosomes are not properly attached. The kinetochore should therefore be able to accurately sense the status of microtubule attachment and interact with the spindle checkpoint components accordingly. How this is achieved is still poorly understood.

## Spindle Checkpoint Proteins at the Kinetochore

While a unifying description of how the kinetochore talks to the checkpoint proteins is still lacking, studies by many different labs lead to several working models on how the checkpoint components interact with the kinetochore and each other to generate inhibitory signals. Currently the most widely accepted model is the conformational change (Mad2-template) model [5], which is based on the structures of different conformations of Mad2 [16-19], structural analysis of the Mad1-Mad2 complex [18], and imaging the dynamics of checkpoint proteins at the kinetochore [20-24]. In this model, free Mad2 exists in an open conformation (O-Mad2) that is unable to interact with Mad1 and Cdc20. In the absence of microtubule attachment, the kinetochore recruits Mad1 dimers, which in turn bind Mad2 (**Figure 1-3**). This leads to the formation of a Mad1-Mad2 complex with Mad2 in the closed conformation (C-Mad2), which wraps around Mad1. The complex recruits free open Mad2 and facilitates its conversion into closed Mad2 and association with Cdc20. The conformational change is important since the closed conformation of Mad2 is required for the binding and inhibition of Cdc20 [23, 25]. The Mad1-Mad2 complex is thought to be essential for “priming” the conversion of Mad2 from open to closed form and thus allows the production of a closed Mad2-Cdc20 complex [26]. The Mad2-Cdc20 complex acts as a diffusible signal and ultimately leads to inhibition of APC activity (discussed below).





**Figure 1-3.** The Mad2-template model. Mad1 dimers associate with unattached kinetochores and bind Mad2, converting them from “open” (O-Mad2) to “closed” (C-Mad2) conformation. The Mad1-Mad2 complexes at the kinetochores (the “templates”) then recruit free open Mad2, facilitating the formation of closed Mad2-Cdc20 complexes. The Mad2-Cdc20 complex acts as a diffusible checkpoint signal from the kinetochore and leads to the inhibition of APC.

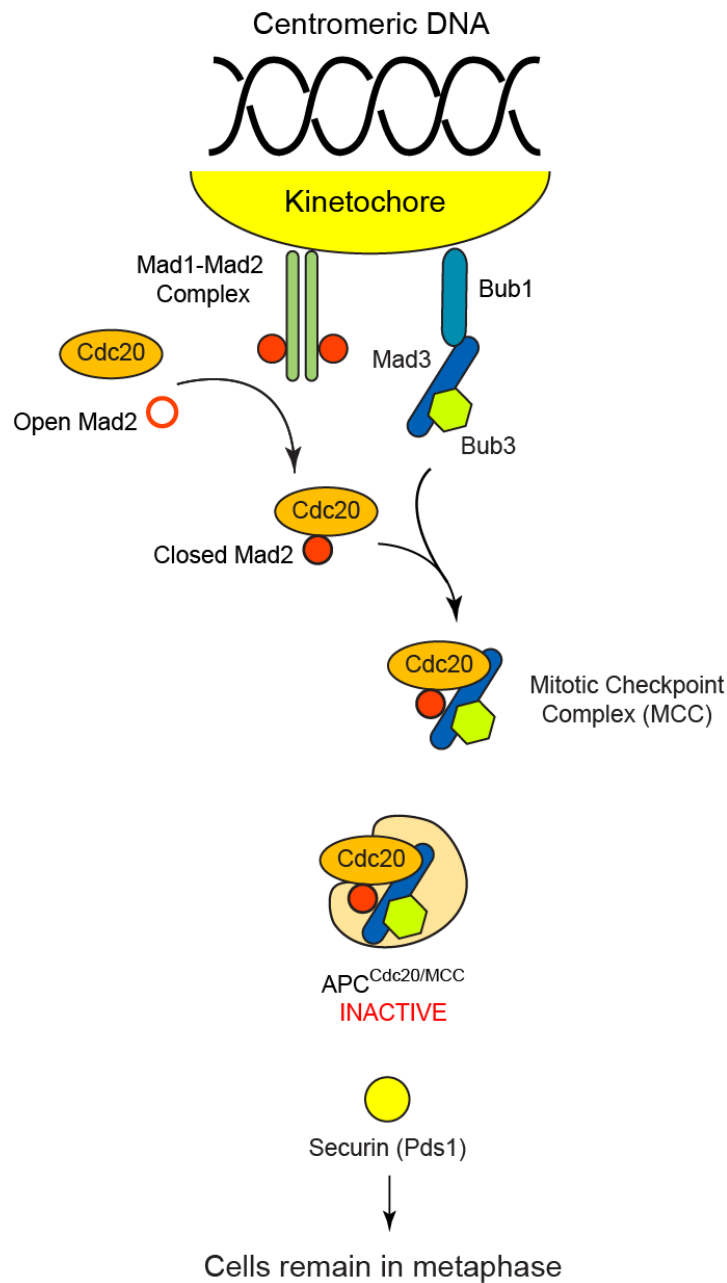
One important extension from the Mad2-template model is that the closed Mad2-Cdc20 complex can recruit open Mad2 and trigger the production of additional closed Mad2-Cdc20, thereby amplifying the checkpoint signal [5]. This can potentially explain how a single improperly attached kinetochore can generate a strong enough signal to completely inhibit APC<sup>Cdc20</sup> activity. But our work using a system that allows Mad2 to associate constitutively with Cdc20 suggests that a Mad2-Cdc20 complex is unable to further inhibit free Cdc20 (discussed in Chapter 4). An alternative mechanism is hence required to ensure complete inhibition of APC<sup>Cdc20</sup> during normal checkpoint activation, and identification of such mechanism will help us understand how even a single kinetochore can lead to cell cycle arrest.

Relatively little is known about how the Mad1-Mad2 complex interacts with the unattached kinetochore. Depletion of subunits of the kinetochore Ndc80 complex by RNAi affects the kinetochore association of Mad1, Mad2, and Mps1 [27, 28], indicating that the Ndc80 complex may be the kinetochore “receptor” that binds the Mad1-Mad2 complex. Forcing the localization of Mad1 to the kinetochore in human tissue culture cells lead to recruitment of Mad2 and metaphase arrest [29], supporting the idea that association between Mad1 and the kinetochore is an integral part of the checkpoint pathway.

Besides Mad1 and Mad2, other checkpoint proteins also have extensive interactions among each other and with the kinetochore. Bub1 has a relatively stable association with the kinetochore [22]. Localizing the checkpoint kinase Mps1 (Mph1 in fission yeast) to the kinetochore is sufficient to recruit Bub1 and activate the spindle checkpoint in fission yeast [30], showing that Mps1 may play a role in Bub1 recruitment

by phosphorylating certain kinetochore components. One possible Mps1 target is the kinetochore protein Blinkin (Spc105 in budding yeast), which is important for the kinetochore recruitment of Bub1 and BubR1 (the mammalian equivalent of Mad3) and checkpoint activation [31]. Localizing Bub1 to an ectopic site on the chromosome in fission yeast leads to recruitment of both Bub3 and Mad3, but fails to arrest the cell cycle [32]. Bub1 may therefore support the formation of a Bub1-Bub3-Mad3 complex at the kinetochore that is important but not sufficient for downstream checkpoint signaling.

Based on the above findings, we favor a “kinetochore scaffold” model to explain how kinetochore leads to spindle checkpoint activations [33]. Both Mad1 and Bub1 associate with unattached kinetochores and act as the main scaffolds. They recruit Mad2, Mad3, and Bub3 to the kinetochore, forming a Mad1-Mad2 and a Bub1-Bub3-Mad3 complex (**Figure 1-4**). Bub1 associates with Mad1 in budding yeast [34], suggesting that the two complexes can potentially interact. Concentrating different checkpoint proteins together at the kinetochore may lead to formation of additional inhibitory complexes. One example is the mitotic checkpoint complex (MCC), which is proposed to consist of Mad2, Mad3, Bub3 and Cdc20 and has been shown to be a potent inhibitor of APC<sup>Cdc20</sup> [35, 36] (**Figure 1-4**). The kinetochore thus acts as a hub for the recruitment of different checkpoint proteins to allow the production of inhibitory complexes. Certain components at the kinetochore may also modify the bound checkpoint proteins to further stimulate the inhibitory signals. In vitro experiments showed that purified kinetochores on chromosomes can stimulate MCC production [37], supporting the idea that the kinetochore is important for the efficient formation of downstream checkpoint effectors.



**Figure 1-4.** The kinetochore scaffold model. Mad1 and Bub1 associate with unattached kinetochores and act as the scaffolds. They recruit Mad2, Mad3, and Bub3 to the kinetochore, leading to the formation of a Mad1-Mad2 and a Bub1-Bub3-Mad3 complex. The two complexes may interact through the association between Mad1 and Bub1. Recruiting different checkpoint proteins to the kinetochore allows the formation of downstream inhibitory complexes, such as the mitotic checkpoint complex (MCC). The MCC consists of Mad2, Mad3, Bub3 and Cdc20 and is a potent inhibitor of APC<sup>Cdc20</sup>. Inhibition of APC<sup>Cdc20</sup> stabilizes securin and arrests cells in metaphase in the presence of improperly attached kinetochores.

For proper checkpoint response, the formation of inhibitory complexes must be carefully regulated and should only happen in the absence of proper microtubule attachment. One way to achieve this regulation is to control the interaction between the Mad1 and Bub1 scaffolds, which is important for the downstream events in the spindle checkpoint pathway. Bub1 associates with the kinetochore stably [22], and Mad1 may only be able to bind Bub1 when microtubules are not properly attached to the kinetochore. The presence of microtubule at the kinetochore may physically block the interaction between Mad1 and Bub1. Microtubule binding may also lead to post-translational modifications or structural changes to Bub1 and affect its binding to Mad1. In addition, microtubule attachment can change the overall structure of the kinetochore [38], which may further prevent Bub1 from associating with Mad1. Understanding the exact events at the kinetochore induced by microtubule attachment and their effects on checkpoint proteins recruitment and interaction will be an important area of future research.

Another important aspect of the spindle checkpoint is that cells must be able to quickly recover from the checkpoint-induced arrest when all chromosomes are properly attached. Most studies have focused on how the cells get rid of the inhibitory checkpoint complexes, such as closed Mad2-Cdc20, once all the chromosomes are properly aligned. One possibility is that closed Mad2-Cdc20 complexes rapidly disassemble in the absence of Mad1-Mad2 complexes at the kinetochores, and an intrinsically fast off rate of closed Mad2 from Cdc20 is sufficient to allow cells to rapidly progress through the cell cycle once all kinetochores are attached. Cells may also have other mechanisms to actively silence the checkpoint. In animal cells, p31-comet is implicated for inactivating the

checkpoint by binding to closed Mad2, which may prevent further production of closed Mad2-Cdc20 complex or compete with Mad3 for binding to Mad2 and thus inhibiting assembly of the MCC [39-41]. Since p31-comet is not found in budding yeast, additional experiments are clearly needed before we can have a clear picture of how cells recover from the spindle checkpoint.

### **Inhibition of APC Activity**

The ultimate goal of the spindle checkpoint is to inhibit the activity of APC<sup>Cdc20</sup>. APC is a large multi-subunit E3 ubiquitin ligase that is essential for cell cycle progression by mediating ubiquitin-dependent degradation of different substrates (reviewed in [42]). The substrates of APC usually contain two destruction motifs; the D box (RxxLxxI/VxN) and the KEN motif (KENxxxN/D) [43-45]. The activity and substrate specificity of APC depends on its interactions with the co-activators Cdc20 and Cdh1 [46-49]. APC<sup>Cdc20</sup> is the main driving force for metaphase-to-anaphase transition by marking securin for destruction [50], thus breaking the connection between sister chromatids and allowing separation of the chromosomes. Inhibition of APC activity by the spindle checkpoint stabilizes securin, arrests cells in metaphase, and therefore prevents premature chromosome segregation.

The mitotic checkpoint complex (MCC), made up of Mad2, Mad3, Bub3, and Cdc20, is considered to be the major inhibitor of APC activity. Several mechanisms have been proposed to explain the action of MCC. Mad2 and Mad3 promote Cdc20 degradation during checkpoint activation [51, 52], which can interfere with APC activity. In addition, MCC can act as a pseudosubstrate of APC through the N-terminal KEN box

motif in Mad3 [53-55] and prevents proper binding of substrates. A recent crystal structure of the MCC from fission yeast shows that the KEN box-binding site of Cdc20 is occupied by Mad3, while the D box-binding site is oriented in a way that will also affect substrate binding [41]. The binding of Mad2 to Cdc20 can also potentially affect the interactions between Cdc20 and APC that are necessary for stimulating the catalytic activity of APC [41, 56]. Moreover, a model of MCC bound to APC derived from electron microscopy reveals that APC adopts a more closed rigid conformation when bound by MCC [57], which may further results in lower APC activity. It remains to be determined how the different mechanisms coordinate with each other to completely inhibit the activity of APC<sup>Cdc20</sup>.

### **Closing Remarks**

The spindle checkpoint is important for accurate chromosome segregation, a process that has to be tightly regulated to prevent disastrous results to the cells. Experiments employing many different techniques such as fluorescent microscopy and in vitro biochemical reconstitution have significantly advanced our understanding of the system. A complete picture of how the spindle checkpoint work will require additional studies to address questions such as the exact molecular events that allow the kinetochore to generate signals to inhibit cell cycle progression, which hopefully will be an achievable goal in the near future.

In Chapter Two of this thesis, I will summarize my attempt to test the “kinetochore scaffold” model by recruiting checkpoint proteins to the chromosome. The results from these experiments were confusing and often hard to interpret. Follow-up

experiments revealed that dimerizing the checkpoint protein Bub1 leads to spindle checkpoint activation, which I will discuss in Chapter Three. This work in turn promoted me to try fusing different checkpoint proteins together, and in Chapter Four I will describe how I concluded from these experiments that Mad2 and Mad3 act as one of the most downstream components in the checkpoint pathway by binding to Cdc20.



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## **Chapter Two**

### **Recruiting Spindle Checkpoint Proteins to the Chromosome**

## Abstract

Correct chromosome segregation during mitosis depends on the spindle checkpoint, which monitors microtubule attachments at the kinetochore and arrests cells before the metaphase-to-anaphase transition until all chromosomes are properly attached. It is not clear how events at the kinetochore activate the spindle checkpoint and induce cell cycle arrest. To test the idea that the kinetochore leads to checkpoint activation by acting as a scaffold to localize checkpoint proteins, we artificially recruited different checkpoint components to the chromosome using binding of the lactose repressor (LacI) to the lactose operator (LacO). Cells carrying LacO repeats arrested in metaphase when they also expressed protein fusions of LacI fused to Bub1 and a mutant version of Bub3 (*BUB3-A117T*). The results suggest that localizing Bub1 and *BUB3-A117T* to the chromosome is sufficient to activate the checkpoint, potentially by further recruiting other checkpoint components. Additional experiments showed that a small fraction of cells that did not have any LacO DNA activated the spindle checkpoint when they only expressed Bub1-LacI, which argues that Bub1-LacI can induce metaphase arrest even when it is not recruited to the chromosome.

## Introduction

Segregation of genetic materials is an integral part of the cell division cycle. In eukaryotes, DNA replication produces two identical copies of chromosomes, which are separated from each other in mitosis. Chromosome segregation relies on the pulling force of microtubules, which attach to the chromosome by binding to a protein complex called the kinetochore. When all chromosomes are attached by microtubules, the anaphase-promoting complex (APC) and its co-activator Cdc20 catalyze the ubiquitination and destruction of securin, triggering anaphase and chromosome segregation. Any mistakes during these processes are likely detrimental. Cells are thus equipped with different mechanisms to ensure that all the steps are carried out properly and in the correct order.

The spindle checkpoint is essential for accurate chromosome segregation by making sure that all chromosomes are lined up on the mitotic spindle before the cells enter anaphase. The spindle checkpoint consists of proteins including Mad1, Mad2, Mad3 [1], Bub1, Bub3 [2], Mps1 [3], Ipl1 [4], and Sgo1 [5], which were first identified by screening for budding yeast mutants that fail to respond to microtubule attachment errors. When even a single kinetochore is not properly attached, the spindle checkpoint is activated and stops cell cycle progression by inhibiting the activity of APC<sup>Cdc20</sup>. We still do not understand how events at the kinetochore signal to the spindle checkpoint and subsequently inhibit APC activity. One model is that in the absence of microtubule attachment, the kinetochore acts as a hub and recruits different spindle checkpoint proteins, ultimately facilitates the formation of inhibitory checkpoint complexes and leads to cell cycle arrest (the “kinetochore scaffold” model, discussed in Chapter One).



A prediction from the kinetochore scaffold model is that artificially forcing the localization of checkpoint proteins to the chromosome should mimic events at the unattached kinetochore, thus resulting in the formation of inhibitory complexes and checkpoint activation. Previous studies have looked at the effect of recruiting checkpoint proteins to different locations in the cells. Localizing Bub1 to the telomeres in fission yeast is sufficient to recruit Bub3 and Mad3, showing that Bub1 acts as a scaffold protein [6]. The recruitment of these checkpoint proteins to the telomeres has no effect on cell cycle progression, suggesting that additional components such as Mad1 and Mad2 are required to generate a checkpoint signal [6]. Recruiting the checkpoint kinase Mps1 (Mph1 in fission yeast) to the kinetochore by fusing it to a core kinetochore protein induces the localization of Bub1 and cell cycle arrest in fission yeast cells [7]. Similarly, localizing Mad1 to the kinetochore in human tissue culture cells leads to recruitment of Mad2 and metaphase arrest [8], indicating that targeting Mad1 to the kinetochore is sufficient to activate the spindle checkpoint. These studies support the idea that localization of checkpoint proteins plays a crucial role in initiating the events in the spindle checkpoint pathway.

To look at the effect of localizing checkpoint proteins to an ectopic site in budding yeast, we developed a system that allows us to recruit different checkpoint components to the chromosome by using the binding of the lactose repressor (LacI) to the lactose operator (LacO). We showed that cells carrying LacO repeats arrested in metaphase when they also expressed LacI fused to Bub1 and a mutant version of Bub3 (*BUB3-A117T*). The recruitment of Bub1 and *BUB3-A117T* to the chromosome may thus recruit other checkpoint components and activate the spindle checkpoint. Interestingly,

cells expressing Bub1-LacI alone (without LacO or *BUB3-A117T*-LacI) still showed metaphase arrest, which suggests that Bub1-LacI is able to induce checkpoint activation without being localized to the chromosome.

## Results

### A System for Recruiting Checkpoint Proteins to the Chromosome

Recruitment of checkpoint proteins to an unattached kinetochore may represent an important step in checkpoint activation. To test this idea, we developed a system to determine the effect of artificially localizing checkpoint proteins to the budding yeast chromosome by exploiting the specific binding of the lactose repressor (LacI) to the lactose operator (LacO). We generated protein fusions of different checkpoint proteins (Mad1, Mad2, Mad3, Bub1 and Bub3) fused to the N terminus of LacI. We placed the constructs under the *HIS3* promoter, which is expressed constitutively at a low level but can be further induced by growing cells in medium that lacks histidine and contains the compound 3-aminotriazole [9]. We also integrated a DNA array of 256 LacO repeats into the budding yeast chromosome III. When a LacI protein fusion is expressed in cells carrying the LacO repeats, the protein can be recruited to the chromosome through the binding of LacI to LacO.

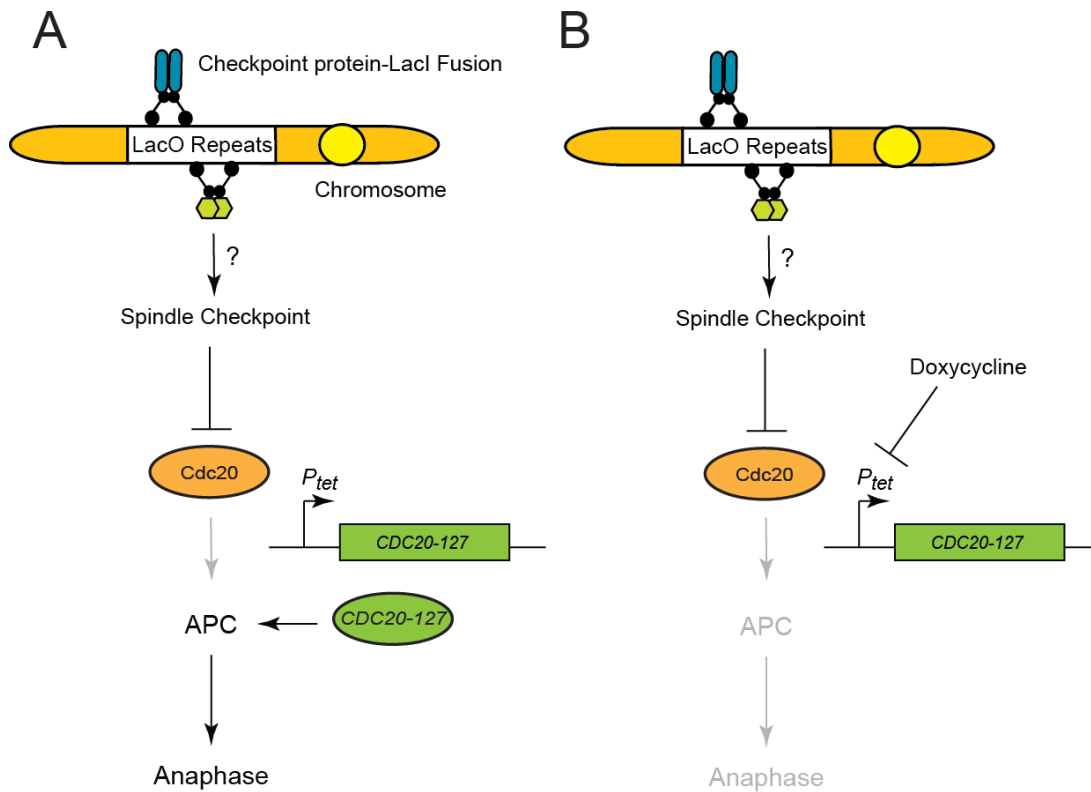
Since the LacI protein fusions are constitutively expressed, cells will fail to proliferate if recruiting the LacI fusions to the chromosome is able to induce metaphase arrest. To circumvent this problem, we introduced a dominant allele of Cdc20 (*CDC20-127*) that the spindle checkpoint cannot inhibit [10]. The allele is expressed from a tetracycline-regulated promoter ( $P_{tet}$ ). In the absence of the drug doxycycline, *CDC20-*

*127* is expressed, which overrides the checkpoint and allows cells to proliferate even when the checkpoint is activated (**Figure 2-1A**). Expression of the allele can be repressed by adding doxycycline, and we can then determine whether recruitment of certain checkpoint proteins will activate the spindle checkpoint (**Figure 2-1B**).

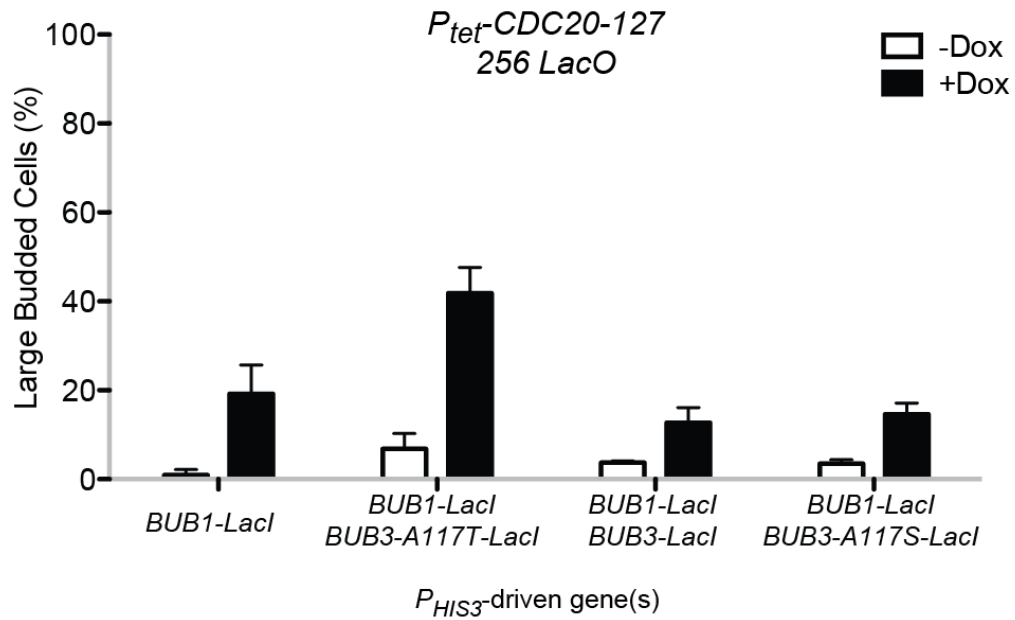
### **Recruiting Bub1 and *BUB3-A117T* to the Chromosome Arrests Cells in Metaphase**

To systematically test the effect of recruiting checkpoint components to the chromosome, we introduced either one or two different LacI protein fusions into strains with 256 LacO repeats and *P<sub>ter</sub>-CDC20-127*. We then asked what will happen to yeast cells going through a synchronous cell cycle when the expression of the checkpoint-resistant *CDC20-127* is repressed. We arrested the cells in G<sub>1</sub> with  $\alpha$ -factor, then released them into rich media with or without doxycycline, and looked at them four hours later. We used the morphology of the cell as an indicator of cell cycle position. If cells activate the checkpoint and arrest in metaphase, they will be enlarged and have large buds similar to the size of the mother cells. On the other hand, cells that are cycling normally have no bud or buds that are smaller than the mother cell.

Most strains that we tested had no obvious phenotype. Almost all the cells had no or small buds when *CDC20-127* was repressed (data not shown). On the other hand, cells with 256 LacO repeats and which expressed a Bub1-LacI protein fusion showed a mild metaphase arrest. When doxycycline was added to the media (to inhibit *CDC20-127* expression), around 20% of the cells were large budded, an indicator of metaphase arrest (**Figure 2-2**). Growing cells in synthetic medium that does not contain histidine and with 3-aminotriazole added (to increase expression of Bub1-LacI) led to a slightly stronger



**Figure 2-1.** Experimental set up to test the effect of recruiting checkpoint proteins to the chromosome. **(A)** Protein fusions of different checkpoint proteins fused to the N terminus of LacI were introduced into yeast cells carrying a DNA array of 256 LacO repeats on chromosome III. The expressed protein fusions are recruited to the chromosome through the specific binding of LacI to LacO. The cells also carry a dominant allele of Cdc20 (*CDC20-127*) that the spindle checkpoint cannot inhibit under a tetracycline-regulated promoter ( $P_{tet}$ ). In the absence of the drug doxycycline, *CDC20-127* is expressed, which allows cells to progress through anaphase even when the spindle checkpoint is activated. **(B)** When doxycycline is added to the media, the expression of *CDC20-127* is inhibited. Cells will arrest in metaphase if the recruitment of checkpoint proteins activates the spindle checkpoint and inhibits Cdc20.



**Figure 2-2.** Recruiting Bub1 and *BUB3-A117T* to the chromosome induces metaphase arrest in cells. Cells with 256 *LacO* repeats on chromosome III, *P<sub>tet</sub>-CDC20-127*, and the indicated *P<sub>HIS3</sub>*-driven genes were released from G<sub>1</sub> arrest into rich media without (-Dox) or with (+Dox) the drug doxycycline (to turn off expression of the checkpoint resistant *CDC20-127*). The percentage of large budded cells was determined by light microscopy after 4 hours of growth. Error bars represent the standard deviation of three independent trials. Two hundred cells were counted for each trial.

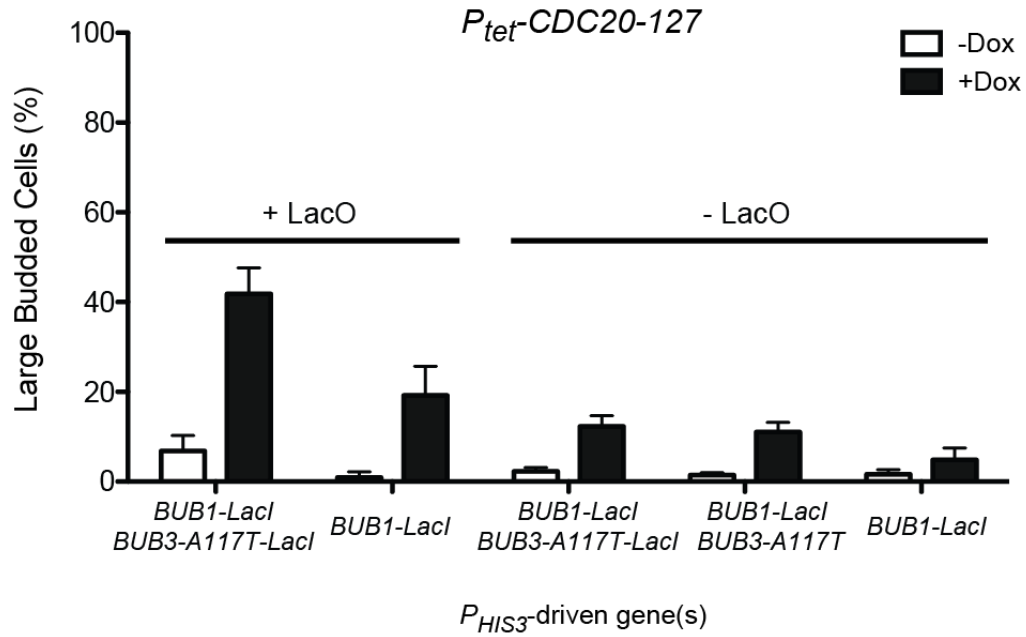
phenotype (data not shown), showing that the metaphase arrest depends on the concentration of Bub1-LacI. We noticed that the cells grew much slower and sometimes had strange morphologies in this case, therefore we decided to use rich media for all the subsequent experiments.

The metaphase arrest induced by Bub1-LacI in cells carrying 256 LacO repeats was much stronger when *BUB3-A117T*-LacI, a mutant version of Bub3-LacI generated by lucky PCR mutation in the cloning process, was also expressed (**Figure 2-2**). In this case more than 40% of the cells were large budded in the presence of doxycycline. Expressing *BUB3-A117T*-LacI alone in cells with LacO repeats had no obvious phenotype (data not shown), suggesting that the stronger arrest is due to the mutant Bub3 potentiating the checkpoint activation by Bub1-LacI. The effect was not observed when wild type Bub3-LacI was expressed instead (**Figure 2-2**), showing that A117T may be a gain-of-function mutation that enables Bub3 to strengthen the metaphase arrest. The crystal structure of the budding yeast Bub3 shows that it is a seven-bladed  $\beta$ -propeller and interacts with Bub1 and Mad3 through its top surface [11, 12]. The A117T mutation is located on one of the  $\beta$  sheets and should not significantly affect the overall structure of Bub3 or its interaction with Bub1 and Mad3. Alternatively, the mutation from alanine to threonine may create a site for phosphorylation. Expressing *BUB3-A117S*-LacI, which should mimic phosphorylation of Bub3 at position 117, had little effect on the level of checkpoint activation in cells with Bub1-LacI and LacO repeats. It is therefore unlikely that the A117T mutation alters Bub3 function by allowing it to be phosphorylated. Additional experiments will be required to determine what effect the mutation may have on Bub3 function.

## **LacO Repeats Strengthen Checkpoint Activation by Bub1-LacI but are not Essential for the Arrest**

A model to explain the results so far is that when Bub1 is localized to the chromosome through the binding of LacI to LacO, it is able to act as a scaffold and recruits other checkpoint proteins such as Bub3 and Mad3 to the site. This is sufficient to activate the spindle checkpoint in a fraction of the cells. The metaphase arrest becomes much more pronounced in the presence of *BUB3-A117T*, which through some as yet unknown mechanisms is able to strengthen checkpoint activation. Our data therefore supports the idea that localizing checkpoint proteins represent an important step in the spindle checkpoint pathway.

If the metaphase arrest we observed is due to localizing Bub1 to DNA, it should depend on the presence of LacO repeats on the chromosome. Even without LacO DNA, around 5% of cells expressing only Bub1-LacI arrested in metaphase (**Figure 2-3**). The result indicates that Bub1-LacI can activate the checkpoint even when it is not recruited to the chromosome, likely through a mechanism that does not require its ability to bind LacO. The phenotype is slightly stronger when *BUB3-A117T*-LacI or untagged *BUB3-A117T* was also expressed (**Figure 2-3**), showing that the expression of *BUB3-A117T* alone, independent of its localization, can strengthen the metaphase arrest induced by Bub1-LacI.



**Figure 2-3.** The presence of LacO repeats strengthens the metaphase arrest induced by Bub1-LacI by is not absolutely required. Cells with *P<sub>tet</sub>-CDC20-127*, the indicated *P<sub>HIS3</sub>*-driven genes, and with (+LacO) or without (-LacO) 256 LacO repeats were released from G<sub>1</sub> arrest into rich media without (-Dox) or with (+Dox) the drug doxycycline (to turn off expression of the checkpoint resistant *CDC20-127*). The percentage of large budded cells was determined by light microscopy after 4 hours of growth. Error bars represent the standard deviation of three independent trials. Two hundred cells were counted for each trial.



## Conclusions and Discussion

We developed a system for recruiting checkpoint proteins to the chromosome by taking advantage of the specific binding of LacI to LacO. Our initial results suggested that recruiting Bub1 and *BUB3-A117T* to the budding yeast chromosome is sufficient to activate the spindle checkpoint. We still observed metaphase arrest in a small portion of cells when only Bub1-LacI was expressed, indicating that the checkpoint activation is caused by the fusion of Bub1 to LacI and does not completely depend on the recruitment of Bub1 to DNA.

Expressing *BUB3-A117T*-LacI, but not wild type Bub3-LacI, led to a stronger metaphase arrest in cells expressing Bub1-LacI with or without LacO. The mutant Bub3 therefore somehow potentiates the checkpoint activation by Bub1-LacI. This increase in cells arrested in metaphase was observed when untagged *BUB3-A117T* was expressed, showing that the mutant can exert its effect even when it is not fused to LacI. One interesting question is whether *BUB3-A117T* can also strengthen spindle checkpoint activation in response to other perturbations such as microtubule poisons. Replacing endogenous Bub3 with *BUB3-A117T* in wild type yeast cells did not have an obvious effect on the ability of the spindle checkpoint to arrest cells in the presence microtubule depolymerizing drugs (data not shown). It is possible that the mutant can potentiate checkpoint activation but our assay is not sensitive enough to detect the effect. Another important issue is how the mutation affects the function of Bub3. The mutation may affect the structure of Bub3 or alter its interactions with other checkpoint components. Experiments such as coimmunoprecipitation to look at how the mutant Bub3 interacts with other checkpoint proteins will be an interesting future direction.

We conclude from our results that Bub1-LacI is able to activate the spindle checkpoint through mechanisms that are induced by fusing Bub1 to LacI but independent of the ability of the protein fusion to bind LacO. Free Bub1-LacI is sufficient to arrest cells in metaphase, albeit weakly, presumably by interacting with other components of the spindle checkpoint and subsequently leads to checkpoint activation. We speculate that the more pronounced arrest in LacO repeat array-containing cells is due to the ability of the LacO repeats to increase the local concentration of Bub1-LacI by localizing the protein fusion to the chromosome, thus causing a stronger metaphase arrest. Overall our experiments revealed an unexpected effect of fusing Bub1 to LacI, which will be explained in Chapter Three, but did not conclusively address whether recruiting checkpoint proteins to an ectopic region can affect cell cycle progression. Alternative way of localizing checkpoint proteins, such as tethering them directly to kinetochore components, may potentially answer this question.

## Materials and Methods

### Yeast strains and Methods

All strains are derivatives of W303 (*ade2-1 his3-11,15 leu2-3,112 trp1-1 ura3-1 can1-100*), and are listed in **Table 2-1**. Strains were constructed using standard genetic techniques. All media were prepared using established recipes [13], and contain 2% wt/vol of glucose as the carbon source.

### Cell Cycle Analysis by Light Microscopy (*P<sub>ter</sub>-CDC20-127* strains)

To look at the effect of recruiting different checkpoint constructs to the chromosome on cell cycle progression, cells were first grown to mid-log phase ( $10^7$  cells/ml) in YEP with 2% glucose (wt/vol) (YPD). Cells were then arrested in G<sub>1</sub> by adding 10µg/ml  $\alpha$ -factor (Bio-Synthesis, Lewisville, TX) and incubated for 2 hours at 30°C. Cells were washed four times to remove  $\alpha$ -factor and resuspended in YPD with or without 10µg/ml doxycycline (Sigma-Aldrich, St. Louis, MO) to inhibit expression of *CDC20-127*. After growing for 4 hours at 30°C, the cultures were briefly sonicated to separate cells that fail to dissociate completely after division and the percentage of large-budded cells in each sample was determined by light microscopy.

Table 2-1. Strains used in this Chapter.

Strain Name	Genotype
DLY457	<i>MATa P<sub>ter</sub>-CDC20-127@TRP1 P<sub>HIS3</sub>-BUB1-LacI@URA3</i>
DLY465	<i>MATa P<sub>ter</sub>-CDC20-127@TRP1 LacO256@LEU2 P<sub>HIS3</sub>-BUB1-LacI@URA3</i>
DLY472	<i>MATa P<sub>ter</sub>-CDC20-127@TRP1 LacO256@LEU2 P<sub>HIS3</sub>-BUB1-LacI@URA3 P<sub>HIS3</sub>-BUB3-LacI@ADE2</i>
DLY481	<i>MATa P<sub>ter</sub>-CDC20-127@TRP1 LacO256@LEU2 P<sub>HIS3</sub>-BUB1-LacI@URA3 P<sub>HIS3</sub>-BUB3-A117T-LacI@ADE2</i>
DLY482	<i>MATa P<sub>ter</sub>-CDC20-127@TRP1 P<sub>HIS3</sub>-BUB1-LacI@URA3 P<sub>HIS3</sub>-BUB3-A117T-LacI@ADE2</i>
DLY518	<i>MATa P<sub>ter</sub>-CDC20-127@TRP1 LacO256@LEU2 P<sub>HIS3</sub>-BUB1-LacI@URA3 P<sub>HIS3</sub>-BUB3-A117S-LacI@HIS3</i>
DLY552	<i>MATa P<sub>ter</sub>-CDC20-127@TRP1 P<sub>HIS3</sub>-BUB1-LacI@URA3 P<sub>HIS3</sub>-BUB3-A117T@HIS3</i>

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## **Chapter Three**

### **Dimerization of Bub1 Activates the Spindle Checkpoint**

## **Abstract**

We developed a system for recruiting checkpoint proteins to the chromosome to test the model that the kinetochore activates the spindle checkpoint by acting as a hub for binding different checkpoint proteins. We found that cells expressing Bub1-LacI in the presence of LacO repeats arrest in metaphase, which suggests that the localization of Bub1 to the chromosome can activate the spindle checkpoint. Here we show that expression of Bub1-LacI can arrest cells even in the absence of LacO repeats. The phenotype is caused by dimerization of Bub1 when it is fused to LacI. The cell cycle arrest by the Bub1 dimer requires the presence of most checkpoint proteins, suggesting that it is acting upstream in the spindle checkpoint pathway. Expressing the Bub1 dimer affects chromosome bi-orientation, but the metaphase arrest doesn't depend on the presence of functional kinetochores, showing that the Bub1 dimer may activate the checkpoint through multiple mechanisms. We found that Bub1 may exist as a dimer during the normal cell cycle, which suggests that the Bub1 dimer have functional roles in cells and potentially explains why dimerizing Bub1 leads to spindle checkpoint activation.



## Introduction

For a cell to divide, it needs to carefully duplicate and segregate its chromosomes. The chromosomes are first duplicated in synthesis (S) phase. Microtubules then bind to chromosomes by attaching to the two sister kinetochores, allowing the chromosomes to congress to the middle of the cell (metaphase). The anaphase-promoting complex (APC) and its co-activator Cdc20 subsequently catalyze the ubiquitination and destruction of securin (Pds1 in budding yeast), ultimately triggering chromosome segregation (anaphase). The spindle checkpoint, initially identified in the budding yeast [1, 2], prevents chromosome missegregation by allowing entry into anaphase only if all chromosomes are bi-oriented (the two sister kinetochores bind microtubules from opposite spindle poles) (reviewed in [3, 4]). The kinetochore is required to signal to the spindle checkpoint in the presence of attachment errors, potentially by recruiting spindle checkpoint proteins and allowing the formation of inhibitory checkpoint complexes (the “kinetochore scaffold” model, discussed in Chapter One).

We developed a system to test the effect of recruiting checkpoint proteins to DNA by using the binding of the lactose repressor (LacI) to the lactose operator (LacO). We showed that cells carrying LacO repeats arrest in metaphase when they express Bub1-LacI (described in Chapter Two), which suggests that recruiting Bub1 to the chromosome is able to activate the spindle checkpoint. It turned out that cells expressing Bub1-LacI arrest even in the absence of LacO DNA, indicating that the binding of Bub1 to the chromosome is not required to induce cell cycle arrest.

We decided to look at the effect of Bub1-LacI more carefully. We show that the metaphase arrest in cells expressing Bub1-LacI is caused by dimerization of Bub1 when it is fused to LacI. Supporting this idea, expressing other forms of dimerized Bub1 leads to a similar

cell cycle arrest. The arrest by the Bub1 dimer depends on the presence of most other checkpoint proteins, which suggests that it is acting upstream in the spindle checkpoint pathway. Expression of Bub1-LacI affects chromosome bi-orientation in cells, but the Bub1 dimer can induce metaphase arrest independently of functional kinetochores, indicating that the mechanism of checkpoint activation by the Bub1 dimer is complicated. In addition, we show that Bub1 forms dimers in the unperturbed cell cycle independently of other checkpoint proteins, which agrees with the idea that the Bub1 dimer contributes to the initiation of the spindle checkpoint.

## **Results**

### **Expressing the Bub1 Dimer Arrests Cells in Metaphase**

Previous experiments showed that cells expressing Bub1-LacI (Bub1 fused to the N terminus of lactose repressor) at a low level (from the *HIS3* promoter) arrested in metaphase even in the absence of LacO repeats (described in Chapter Two). Expression of Bub1-LacI therefore somehow induces spindle checkpoint activation. To determine the effect of Bub1-LacI, we asked what would happen if we overexpressed Bub1-LacI from the *GALI* promoter in budding yeast cells going through a synchronous cell cycle. We integrated a *P<sub>GALI</sub>-BUB1-LacI* construct into the yeast genome without modifying the endogenous *BUB1* gene. Genes under the *GALI* promoter are expressed in the presence of galactose and inhibited by glucose. We arrested the cells in G<sub>1</sub> with  $\alpha$ -factor and released them into media with either glucose or galactose. We then looked at the cells three hours later. Cells that are going through the cell cycle normally have no bud or buds that are smaller than the mother cell, whereas cells that are delayed in mitosis are enlarged and have huge buds that approach the size of the mother cells. Overexpressing Bub1 had little effect on the cells; almost the entire population continued to

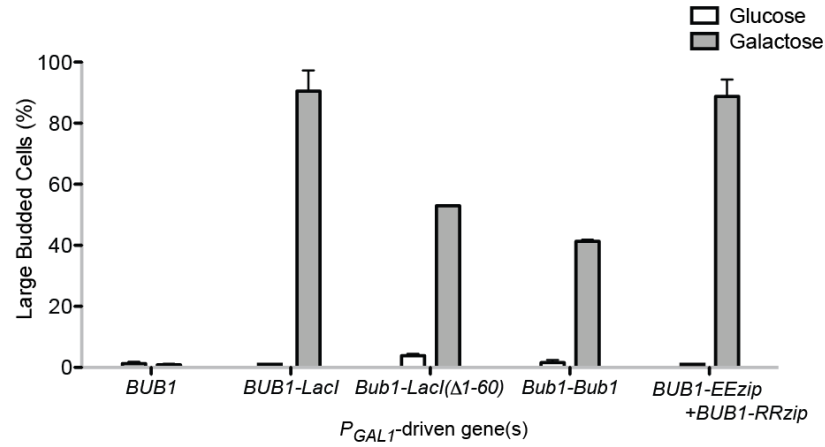
cycle when grown in galactose-containing medium (**Figure 3-1A**). In contrast, overexpressing Bub1-LacI led to accumulation of large budded cells (**Figure 3-1A**), confirming that expression of Bub1-LacI alone can induce checkpoint activation.

We verified that expressing Bub1-LacI activates the spindle checkpoint and inhibits APC activity by using a biochemical marker for the exit into anaphase. Degradation of the APC<sup>Cdc20</sup> substrate securin (named Pds1 in budding yeast) triggers the metaphase-to-anaphase transition. We followed the level of this protein by monitoring the level of Myc-tagged securin (Pds1-18×Myc) on Western Blots. When Bub1-LacI was not expressed (glucose), securin levels rose and fell after cells were released from G<sub>1</sub> arrest, indicating normal cell cycle progression (**Figure 3-1B**). Overexpressing Bub1-LacI (galactose) stabilized securin (**Figure 3-1B**), showing that APC activity is inhibited.

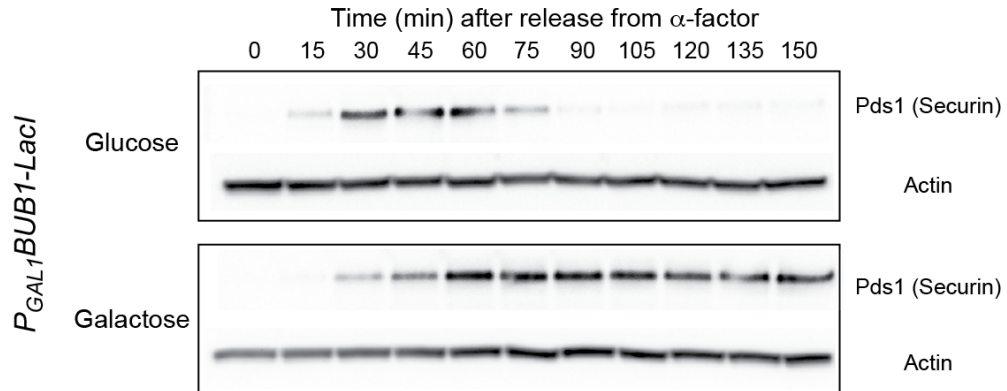
Bub1-LacI can bind DNA non-specifically even in the absence of LacO repeats, and the binding may contribute to checkpoint activation by concentrating Bub1 proteins. To test the requirement for DNA binding, we fused Bub1 to a version of LacI without the N-terminal DNA binding region but retains the dimerization domain (Bub1-LacI( $\Delta$ 1-60)) [5]. Expression of Bub1-LacI( $\Delta$ 1-60) under the *GALI* promoter in synchronized yeast cells again induced metaphase arrest and around 50% of the cells became large budded (**Figure 3-1A**). The DNA binding activity of LacI is thus not required for checkpoint activation by Bub1-LacI.

Another possible effect of fusing Bub1 to LacI is that Bub1-LacI should exist as a dimer through the dimerization domain of LacI. Expression of the Bub1 dimer may be sufficient to arrest cells in metaphase. To test this hypothesis, we generated a covalent Bub1-Bub1 dimer construct with the C terminus of Bub1 fused to the N terminus of another Bub1 by a 36-amino acid linker. Around 40% of the cells expressing Bub1-Bub1 accumulated in the large budded

A



B



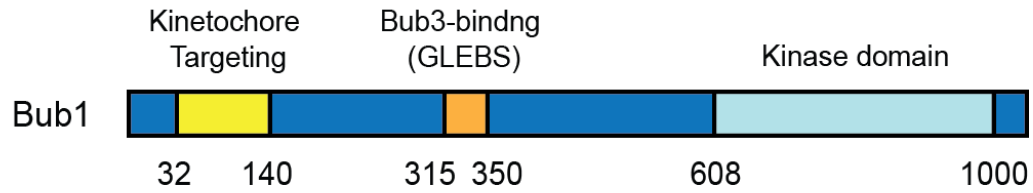
**Figure 3-1.** Expressing the Bub1 dimer arrests cells in metaphase. **(A)** Cells with the indicated  $P_{GAL1}$ -driven genes were grown to mid-log phase, arrested in  $G_1$  with  $\alpha$ -factor, and were released into media with either glucose or galactose. After 3 hours of growth, the percentage of large budded cells was determined by light microscopy as a measure of metaphase arrest. Error bars represent the standard deviation of three independent trials. Two hundred cells were counted for each trial. **(B)** Cell cycle progression of  $P_{GAL1}$ -*BUB1-LacI* cells was monitored by Western blots ( $n=3$ ), which detect securin (Pds1), a protein that is destroyed as cells enter anaphase. Cells were grown to mid-log phase and arrested in  $G_1$  with  $\alpha$ -factor, and were released into media with either glucose (top) or galactose (bottom). At 60 min after release from  $G_1$  arrest,  $\alpha$ -factor was added to prevent cells from progressing into the next S phase. Lysates were prepared from cells harvested at the indicated time and Western blots against Myc (to visualize Myc-tagged securin) or actin (loading control) were performed. When the cells were grown in glucose, securin level first increased and then dropped rapidly as cells progressed into anaphase. When Bub1-LacI is expressed in the presence of galactose, securin was stabilized, indicating that the cells were arrested in metaphase.

state (**Figure 3-1A**), supporting the idea that the Bub1 dimer can induce checkpoint activation. We also used a different, non-covalent method to dimerize Bub1. We fused Bub1 to two engineered leucine zipper sequences (EEzip and RRzip) that allow the formation of stable dimers between the two zippers bearing opposing charges [6]. When we expressed the Bub1 dimer (by releasing cells containing both *P<sub>GALI</sub>-Bub1-EEzip* and *P<sub>GALI</sub>-Bub1-RRzip* into galactose-containing medium), 90% of the cells arrested in metaphase (**Figure 3-1A**). Dimerizing Bub1 can thus activate the spindle checkpoint in cells.

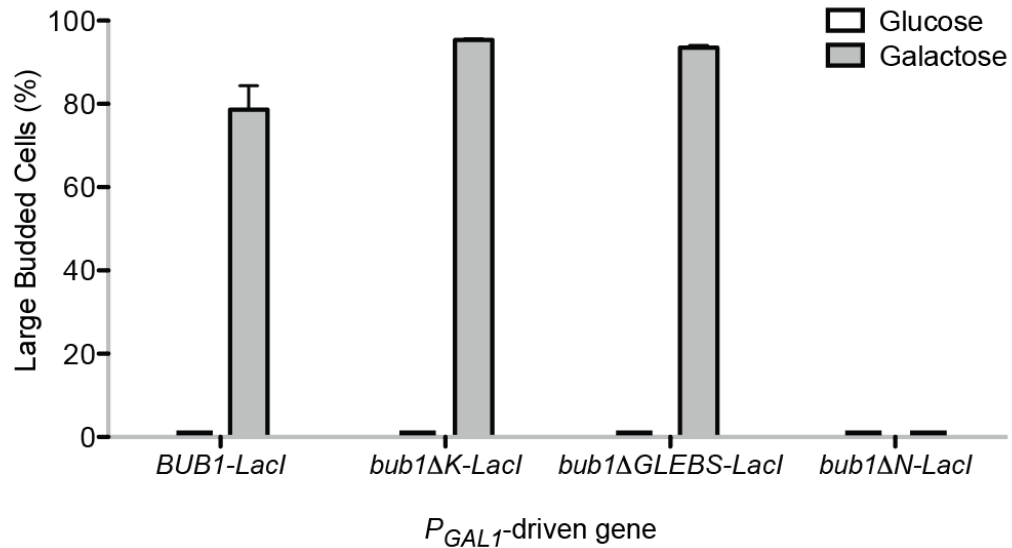
### **Checkpoint Activation by the Bub1 Dimer is Independent of the Kinase and Bub3-binding Domains of Bub1**

Bub1 is a protein kinase that has multiple roles in cells. It contains three main regions: a highly conserved N-terminal region that is important for its kinetochore targeting [7, 8]; a Gle2-binding sequence (GLEBS) domain that is required for Bub3 binding [9]; and a C-terminal region that contains a serine/threonine kinase domain (**Figure 3-2A**). Bub1 is essential for the spindle checkpoint as well as correct partitioning of chromosomes. It has been suggested that Bub1 phosphorylates histone 2A to localize Sgo1, a protein important for chromosome bi-orientation, to the centromere [10-12]. The kinase domain of Bub1 is essential for its role in establishing chromosome bi-orientation but is dispensable for checkpoint function [10-12]. Consistent with this claim, we found that expressing LacI fused to a version of Bub1 that lacks its kinase domain (*bub1 $\Delta$ K-LacI*) can still induce metaphase arrest in cells, showing that the kinase domain of Bub1 is not required for checkpoint activation by the Bub1 dimer (**Figure 3-2B**).

A



B



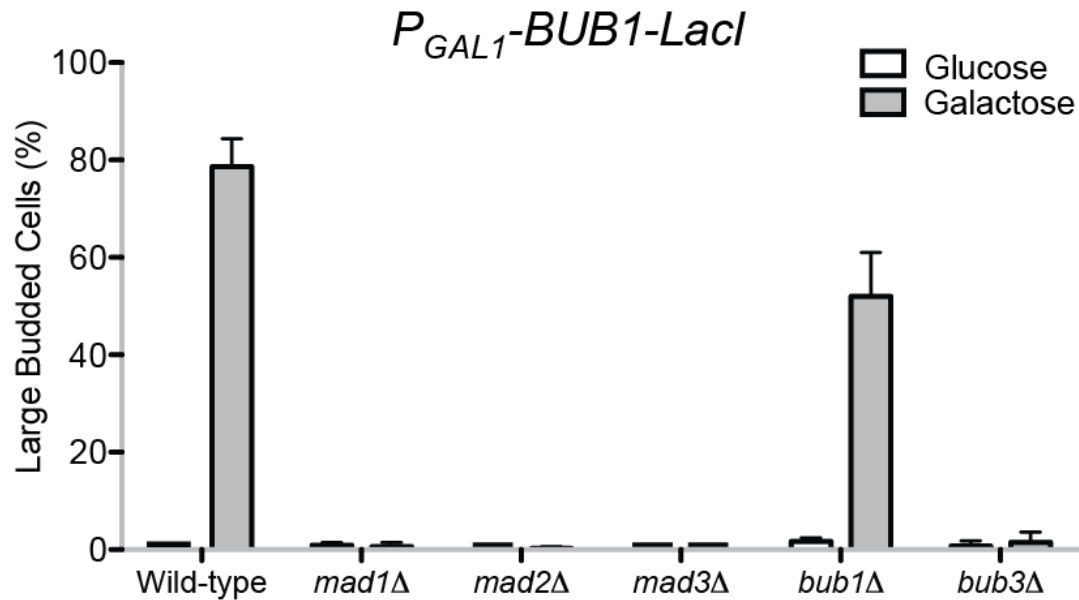
**Figure 3-2.** Metaphase arrest by the Bub1 dimer requires the N-terminal region but not the kinase or the Bub3-binding domain. **(A)** Domain organization of Bub1. Bub1 contains a highly conserved N-terminal region that is important for its targeting to the kinetochore. It also has a Bub3-binding region (GLEBS domain) and a C-terminal kinase domain. **(B)** Cells with the indicated  $P_{GAL1}$ -driven genes were grown to mid-log phase, arrested in G<sub>1</sub> with  $\alpha$ -factor, and were released into media with either glucose or galactose. After 3 hours of growth, the percentage of large budded cells was determined by light microscopy as a measure of metaphase arrest. *bub1ΔK* is a truncated version of Bub1 without the C-terminal kinase domain (containing amino acids 1-608). *bub1ΔGLEBS* and *bub1ΔN* are versions of Bub1 with deletion of amino acids 315-350 and 32-140, respectively. Error bars represent the standard deviation of three independent trials. Two hundred cells were counted for each trial.

We also determined whether other domains of Bub1 are needed for the metaphase arrest by the Bub1 dimer. More than 90% of cells expressing LacI fused to Bub1 without the GLEBS domain (*bub1ΔGLEBS*-LacI) arrested in metaphase, suggesting that the physical interaction between Bub1 and Bub3 is not essential for the Bub1 dimer to activate the checkpoint (**Figure 3-2B**). This result agrees with the finding that the GLEBS motif in Bub1 is dispensable for checkpoint function in fission yeast [13]. On the other hand, removing the N-terminal region from Bub1 (*bub1ΔN*-LacI) completely abolished checkpoint activation (**Figure 3-2B**), which indicates that the kinetochore-targeting domain plays an important role in arresting cells in metaphase.

### **The Bub1 Dimer Requires Most Spindle Checkpoint Proteins to Induce Metaphase Arrest**

We then asked whether the Bub1 dimer needs other checkpoint components to arrest cells in metaphase. We introduced the *P<sub>GALI</sub>-BUB1-LacI* construct into yeast strains with different spindle checkpoint genes deleted (*mad1Δ*, *mad2Δ*, *mad3Δ*, *bub1Δ*, and *bub3Δ*) and tested the effect of expressing Bub1-LacI by releasing the cells from G<sub>1</sub> arrest into galactose-containing medium. In all the strains except *bub1Δ*, cells failed to activate the checkpoint and were cycling normally (**Figure 3-3**). The results show that the Bub1 dimer requires other spindle checkpoint protein (but not endogenous Bub1) for checkpoint activation.

We also tested the requirement for two spindle checkpoint kinases, Mps1 and Ipl1, which have other essential roles in cells. Mps1 is important for the spindle checkpoint as well as the duplication of the spindle pole bodies (SPBs) and mitotic spindle assembly [14, 15]. Ipl1/Aurora B is required to activate the spindle checkpoint in the absence of mechanical tension at the kinetochores [16, 17]; it is also involved other process such as spindle disassembly [18]. Since



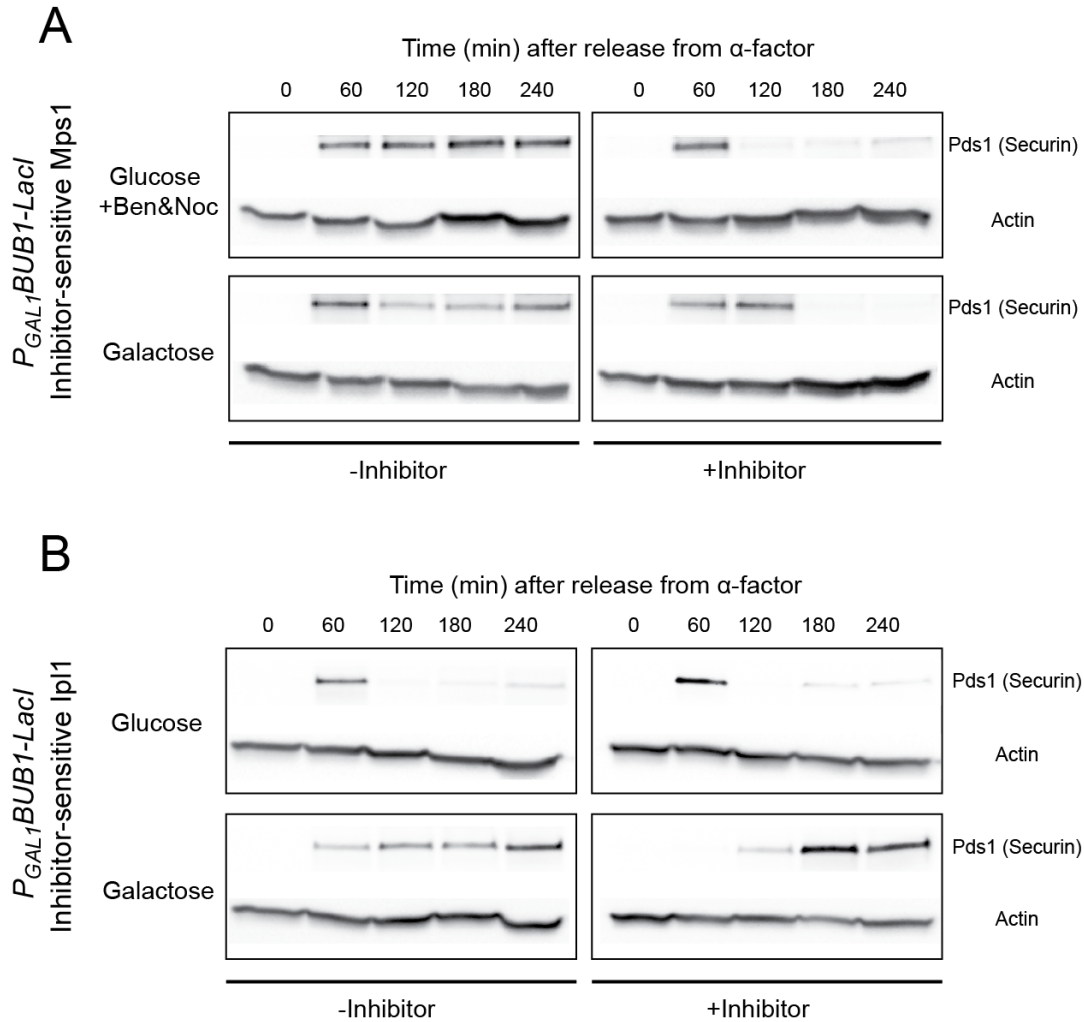
**Figure 3-3.** Checkpoint activation by Bub1-LacI requires most checkpoint components. Cells with *P<sub>GAL1</sub>-BUB1-LacI* and deletion of the indicated checkpoint genes were released from G<sub>1</sub> arrest into glucose- or galactose-containing media. The percentage of large budded cells was determined by light microscopy after 3 hours of growth. Error bars represent the standard deviation of three independent trials. Two hundred cells were counted for each trial.



both Mps1 and Ipl1 are essential for cell viability, we used conditional alleles to inhibit their activities to ask whether they are required for Bub1-LacI to arrest cells. For Mps1, we used the analog-sensitive allele *mps1-as1* [15], which can be specifically inhibited by a bulky protein kinase inhibitor [19]. We released *mps1-as1* cells carrying *P<sub>GALI</sub>-BUB1-LacI* from G<sub>1</sub> arrest into media with or without the kinase inhibitor (1NM-PP1) and monitored securin levels. Cells treated with the inhibitor that were grown in glucose-containing medium (to inhibit expression of Bub1-LacI) with benomyl and nocodazole (to depolymerize microtubules) showed a rise and fall in securin (**Figure 3-4A**). This result confirms that Mps1-as1 cannot function and the spindle checkpoint is abolished in the presence of the inhibitor. Cells that were grown in galactose-containing medium (to express Bub1-LacI) also showed a similar rise and fall in securin level (**Figure 3-4A**), indicating that inactivation of Mps1 activity prevents metaphase arrest by Bub1-LacI. We performed similar experiments to test the requirement for Ipl1 by using the analog-sensitive allele, *ipl1-as5* [17]. We released *ipl1-as5* cells carrying *P<sub>GALI</sub>-BUB1-LacI* from G<sub>1</sub> arrest into media with or without a different inhibitor (1NA-PP1). Expressing Bub1-LacI stabilized securin even when Ipl1 was inactivated by the inhibitor (**Figure 3-4B**), indicating that Ipl1 function is not necessary for metaphase arrest by the Bub1 dimer. Overall Bub1-LacI requires most checkpoint proteins to activate the spindle checkpoint, suggesting that it exerts its effect upstream in the checkpoint pathway.

### **Expression of the Bub1 Dimer Affects Chromosome Bi-orientation**

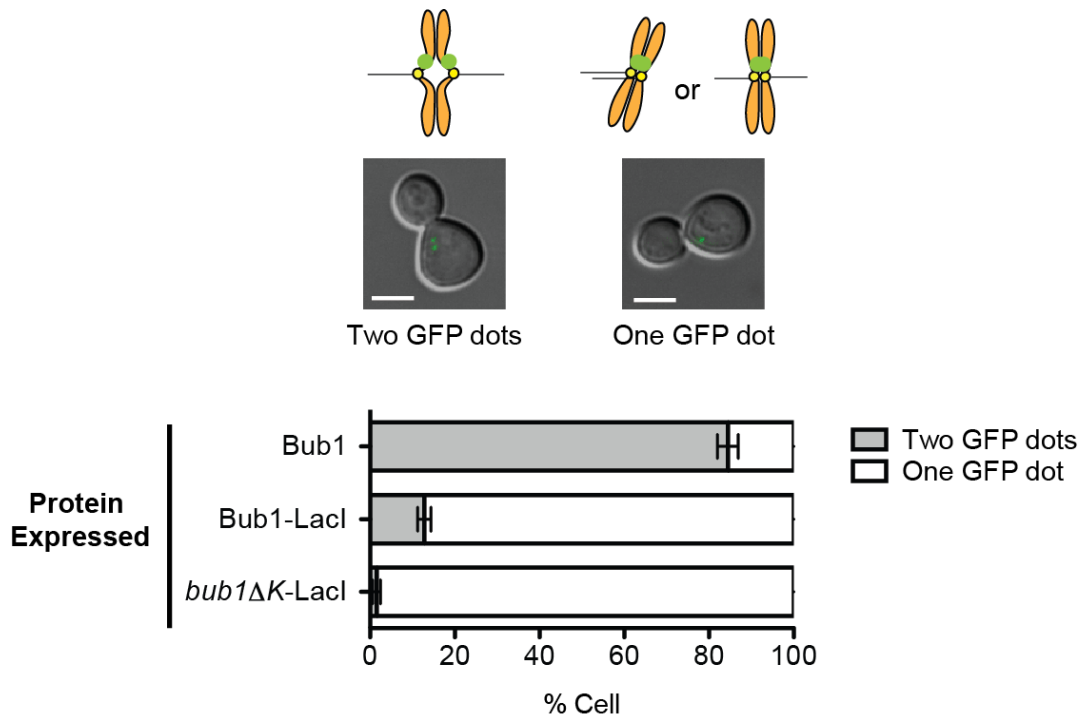
Why does the Bub1 dimer arrest cells in metaphase? One possible explanation is that expression of the Bub1 dimer somehow affects microtubule attachments to chromosomes, and the defects in turn activate the spindle checkpoint. We therefore looked at chromosome bi-



**Figure 3-4.** Spindle checkpoint activation by Bub1-LacI requires Mps1 but not Ipl1 function. **(A)** Cell cycle progression of cells with *P<sub>GAL1</sub>-BUB1-LacI* and *mps1-as1* was monitored by Western blotting (n=3). Cells were released from G<sub>1</sub> arrest into media with either glucose with benomyl and nocodazole (top) or galactose (bottom), in the absence (-Inhibitor) or presence (+Inhibitor) of 1NM-PP1, an inhibitor of the analog-sensitive Mps1. At 60 min after release from G<sub>1</sub> arrest,  $\alpha$ -factor was added to prevent cells from progressing into the next S phase. Lysates were prepared from cells harvested at the indicated time and Western blots against Myc or actin (loading control) were performed. Both securin and Mps1-as1 are tagged with Myc in the strain, and only the bands corresponding to Myc-tagged securin are shown in the figure. Securin level increased and then dropped in galactose-containing medium in the presence of inhibitor, indicating that Mps1 activity is needed for the metaphase arrest. **(B)** The cell cycle progression of cells with *P<sub>GAL1</sub>-BUB1-LacI* and *ipl1-as5* was measured by Western blots (n=3). Cells were released from G<sub>1</sub> arrest into media with either glucose (top) or galactose (bottom), in the absence (-Inhibitor) or presence (+Inhibitor) of 1NA-PP1, an inhibitor of the analog-sensitive Ipl1. At 60 min after release from G<sub>1</sub> arrest,  $\alpha$ -factor was added to prevent cells from progressing into the next S phase. Lysates were prepared from cells harvested at the indicated time and Western blots against Myc (to visualize Myc-tagged securin) or actin (loading control) were performed. Securin was stabilized in galactose-containing medium in the presence of inhibitor, showing that Ipl1 activity is not needed for the metaphase arrest.

orientation in cells at metaphase. A chromosome is bi-oriented when the two sister kinetochores attach to microtubules from opposite spindle poles. The sister kinetochores can be pulled apart from each other by the microtubules, and we can visualize the separation (about 0.5  $\mu\text{m}$ ) by expressing a GFP-LacI and placing a LacO array near the centromere [20-23]. We integrated different Bub1 constructs under the *GALI* promoter into a strain that expresses GFP-LacI, has a LacO array near the centromere of chromosome XV, and CDC20 under the methionine-repressible *MET3* promoter ( $P_{MET3}\text{-CDC20}$ ). We grew the cells in medium with galactose (to express the Bub1 construct) and methionine (to inhibit Cdc20 expression). The cells arrested in metaphase because they cannot activate the APC in the absence of Cdc20. Around 80% of the cells expressing Bub1 in this case showed two GFP dots, indicating that chromosome XV bi-oriented (**Figure 3-5**). The percentage of cells with two GFP dots was much lower (around 15%) when cells expressed Bub1-LacI instead (**Figure 3-5**). Chromosomes bi-orientation is thus affected by Bub1-LacI, showing that the Bub1 dimer potentially activates the spindle checkpoint by creating mono-oriented chromosomes.

Bub1 localizes at kinetochores and is important for chromosome bi-orientation, for example by phosphorylating histone 2A and recruiting Sgo1 to the centromere [10-12]. The kinase activity and kinetochore localization of Bub1 is essential for its role in establishing bi-oriented chromosomes. We speculate that when the Bub1 dimer is expressed at a high level, a portion of the dimers fail to localize to the kinetochore. The mislocalized Bub1 dimers may interfere with chromosome bi-orientation by sequestering downstream targets away from Bub1 at the kinetochore. Cells that were arrested in metaphase by Cdc20 depletion had an even stronger defect in chromosome bi-orientation when they expressed *bub1 $\Delta$ K*-LacI (**Figure 3-5**). One explanation is that in addition to binding to Bub1 targets and keeping them away from the

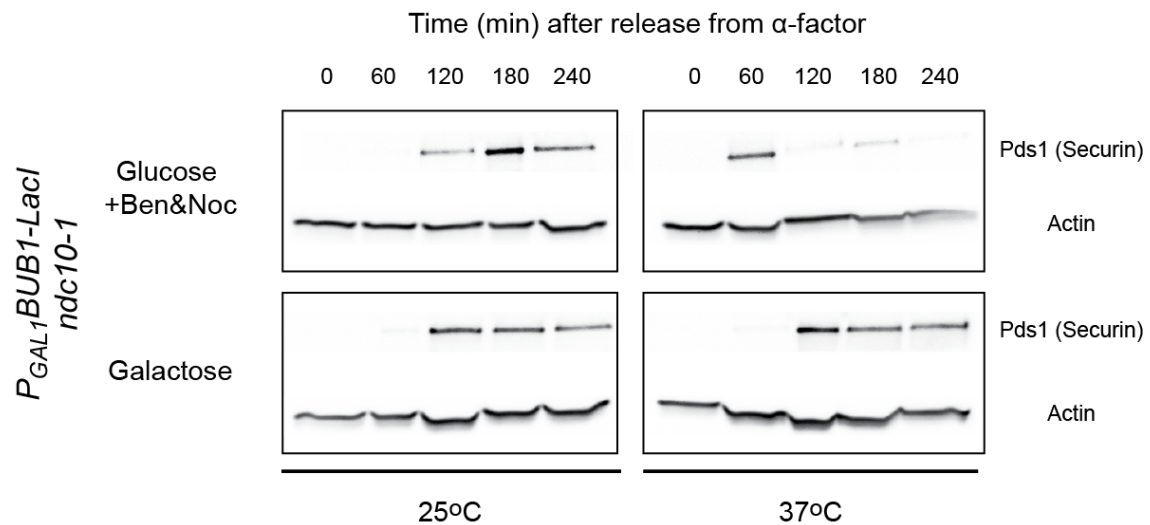


**Figure 3-5.** Expression of Bub1-LacI affects chromosome bi-orientation. To achieve metaphase arrest, cells carrying the indicated  $P_{GALI}$ -driven genes, GFP-LacI, a LacO array located near the centromere of chromosome XV, and  $P_{MET3}$ -*CDC20* were released from G<sub>1</sub> arrest into medium with galactose (to express the checkpoint construct) and methionine (to inhibit Cdc20 expression). Differential interference contrast (DIC) and GFP images of the cells were taken 3 hours after their release from G<sub>1</sub>. One or two GFP dots can be seen in cells arrested in metaphase. Cells have two GFP dots when chromosome XV bi-orient and the two sister kinetochores are separated by the spindle. Cells have one GFP dot when chromosome XV mono-orient (sister kinetochores attaching to the same spindle pole) or bi-orient but the two sister kinetochores are not pulled apart enough to allow resolution of two separate dots. Representative images of metaphase-arrested cells with one or two GFP dots are shown (Scale bar, 5 μm). The bar graph shows the percentage of cells with one or two GFP dots when they were arrested by Cdc20 depletion. Error bars represent the standard deviation of three independent trials. Two hundred cells were counted for each trial. Around 80% of the cells expressing Bub1 had two GFP dots. The percentages of cells that showed two GFP dots when Bub1-LacI or *bub1ΔK*-LacI was expressed were much lower. Chromosome bi-orientation (or the ability to apply force to bioriented chromosomes) is therefore compromised in cells expressing the Bub1 dimer.

kinetochore, Bub1-LacI and *bub1ΔK*-LacI also replace the endogenous Bub1 at the kinetochore. While Bub1-LacI can perform the functions of Bub1 at the kinetochore, *bub1ΔK*-LacI is unable to support chromosome bi-orientation because it lacks kinase activity. Expressing *bub1ΔK*-LacI in cells thus induces a stronger bi-orientation defect.

### **Metaphase Arrest by the Bub1 Dimer Does Not Require Functional Kinetochores**

The presence of functional kinetochores is required to signal to the spindle checkpoint in response to microtubule attachment defects [24, 25]. Metaphase arrest by the Bub1 dimer should depend on kinetochore functions if the Bub1 dimer activates the checkpoint solely by disrupting chromosome bi-orientation. We looked at the phenotype of Bub1-LacI expression in the absence of functional kinetochores by using an *ndc10-1* strain. Ndc10 is a member of the CBF3 complex of the kinetochore, which binds to the centromere and serves as the primary link between the chromosome and microtubule binding complexes of the kinetochore [26]. *ndc10-1* cells lack functional kinetochores at the restrictive temperature (37°C) and are unable to activate the spindle checkpoint even in the presence of the microtubule depolymerizing drugs benomyl and nocodazole [24, 25]. If the Bub1 dimer activates the checkpoint by interfering with chromosome bi-orientation, we should not observe metaphase arrest when Bub1-LacI is expressed in *ndc10-1* cells at 37°C. To test this prediction, we released *ndc10-1* cells carrying *P<sub>GALI</sub>-BUB1-LacI* from a G<sub>1</sub> arrest at 25°C or 37°C and monitored the level of securin. In cells that were released into glucose-containing medium (to inhibit expression of Bub1-LacI) with benomyl and nocodazole (to depolymerize microtubules) at 37°C, securin levels rose and fell, showing that cells failed to activate the spindle checkpoint in the absence of functional kinetochores (**Figure 3-6**). In contrast, when *ndc10-1* cells expressed Bub1-LacI (by releasing them into galactose-containing



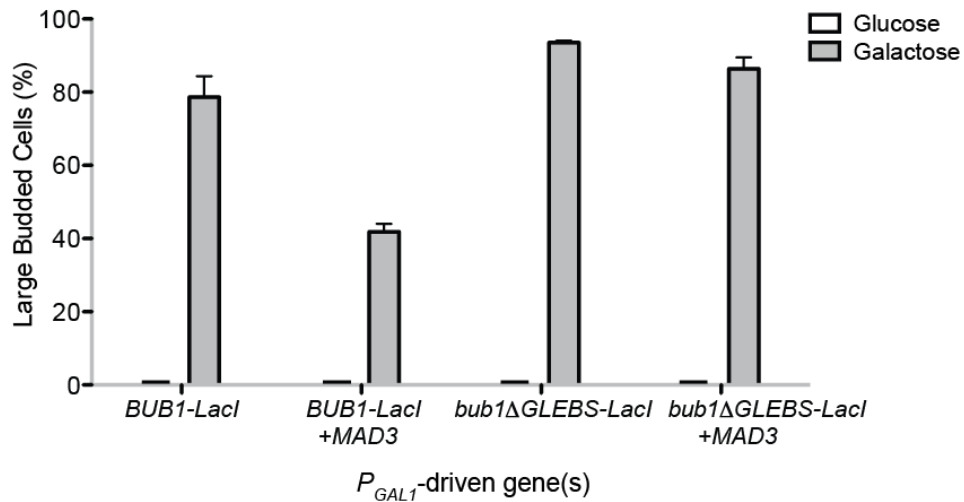
**Figure 3-6.** The metaphase arrest by Bub1-LacI does not require functional kinetochores. Cell cycle progression of cells with  $P_{GAL1}\text{-}BUB1\text{-}LacI$  and  $ndc10\text{-}1$  (a mutation that inactivates kinetochore at 37°C) was measured by Western blots (n=3). Cells were released from  $G_1$  arrest into media with either glucose with benomyl and nocodazole (top) or galactose (bottom) at 25°C or 37°C. At 60 min after release from  $G_1$  arrest,  $\alpha$ -factor was added to prevent cells from progressing into the next S phase. Lysates were prepared from cells harvested at the indicated time and Western blots against Myc (to visualize Myc-tagged securin) or actin (loading control) were performed. Securin was stabilized in galactose-containing medium even at 37°C, indicating that the metaphase arrest by Bub1-LacI is independent of functional kinetochores.

medium) at 37°C, securin was stabilized (**Figure 3-6**). Similar results were observed in *ndc10-1* cells expressing *bub1ΔK*-LacI (data not shown). The Bub1 dimer can thus arrest cells in metaphase even in the absence of functional kinetochores, suggesting that it is able to activate the checkpoint independently of its effect on chromosome bi-orientation.

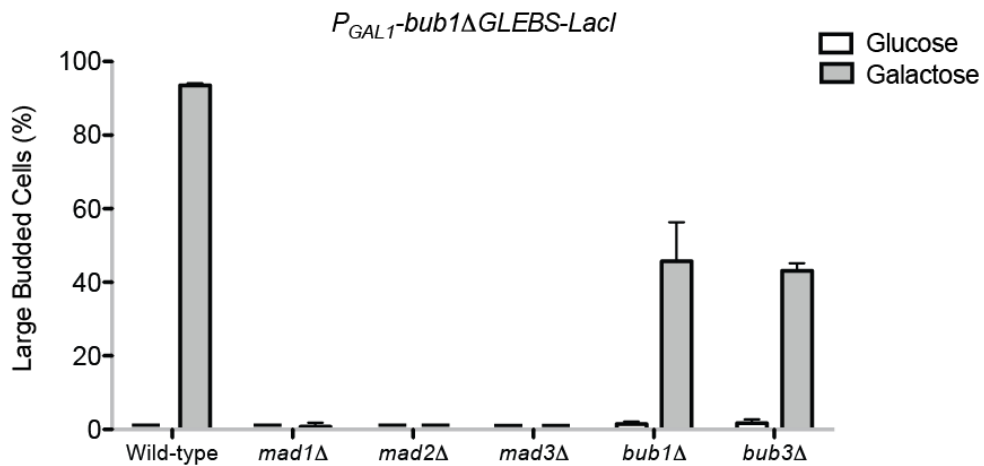
### **A High Level of Mad3 Interferes with Metaphase Arrest by the Bub1 Dimer**

To determine what checkpoint proteins the Bub1 dimer may interact with, we expressed other checkpoint components with Bub1-LacI to see how they affected the level of checkpoint activation. Overexpressing Mad3 together with Bub1-LacI led to a weaker metaphase arrest than expressing Bub1-LacI alone, suggesting that Mad3 interferes with the ability of Bub1-LacI to activate the checkpoint (**Figure 3-7A**). Both Bub1 and Mad3 contain a conserved Gle2-binding sequence (GLEBS) motif which bind the same surface of Bub3 in a mutually exclusive manner [9, 27, 28]. A high concentration of Mad3 may act as a competitive inhibitor and sequester Bub3 away from the Bub1 dimer, therefore affecting the extent of checkpoint activation. Although the interaction between Bub1 and Bub3 is thought to be important for the spindle checkpoint, expressing LacI fused to Bub1 without the GLEBS motif (*bub1ΔGLEBS*-LacI) still arrested cells in metaphase, suggesting that the Bub1 dimer can activate the checkpoint without associating with Bub3 (**Figure 3-2B** and **Figure 3-7A**). Interestingly, the arrest induced by *bub1ΔGLEBS*-LacI was not affected by Mad3 expression (**Figure 3-7A**). While certain checkpoint proteins like Mad3 are required for metaphase arrest by *bub1ΔGLEBS*-LacI, a moderate percentage of cells expressing *bub1ΔGLEBS*-LacI arrested even in the absence of Bub3 (**Figure 3-7B**). One possible explanation for these results is that the GLEBS domain normally inhibits the checkpoint function of Bub1, for example by physically blocking the surface for substrate binding. The binding of

**A**



**B**



**Figure 3-7.** Checkpoint activation by the Bub1 dimer is affected by a high concentration of Mad3 in a Bub3-binding motif dependent manner. **(A)** Cells with the indicated  $P_{GAL1}$ -driven genes were released from G<sub>1</sub> arrest into glucose- or galactose-containing media. The percentage of large budded cells was determined by light microscopy after 3 hours of growth. Error bars represent the standard deviation of three independent trials. Two hundred cells were counted for each trial. **(B)** Cells with  $P_{GAL1}$ -*bub1ΔGLEBS-LacI* and deletion of the indicated checkpoint genes were released from G<sub>1</sub> arrest into glucose- or galactose-containing media. The percentage of large budded cells was determined by light microscopy after 3 hours of growth. Error bars represent the standard deviation of three independent trials. Two hundred cells were counted for each trial.



Bub3 to the GLEBS domain may move the motif away from Bub1, therefore relieving the inhibition and activating the checkpoint function of Bub1. A high concentration of Mad3 sequesters Bub3 and interferes with this process, which explains why overexpressing Mad3 affects the checkpoint activation by the Bub1 dimer. On the other hand, Bub1 is no longer inhibited by the GLEBS domain when the motif is deleted. Bub3 is dispensable for stimulating the checkpoint function of Bub1 in this case. *bub1ΔGLEBS-LacI* can thus arrest cells in metaphase independently of Bub3 and the arrest is not affected by a high level of Mad3. It will be interesting to test whether this model is true by further investigating how Mad3, Bub1, and Bub3 coordinate with each other during checkpoint activation.

### **Bub1 Interacts with Itself During the Yeast Cell Cycle**

Dimerization of Bub1 might occur during the cell cycle as an essential step in spindle checkpoint activation. If so, the expression of the artificial Bub1 dimer simply recapitulates this event and hence activates the checkpoint. Bub1 associates with both Bub3 and Mad1 [29, 30], but it is not clear whether Bub1 also interacts with itself. To look for potential Bub1 dimer, we constructed diploid strains that carry Myc-tagged (Bub1-13×Myc) and FLAG-tagged Bub1 (Bub1-3×FLAG) under the *GALI* promoter and performed coimmunoprecipitation (co-IP) experiments. We grew the cells in media with galactose (to express the Bub1 constructs) either with or without the microtubule depolymerizing drugs benomyl and nocodazole for three hours. We expressed the two epitope-tagged versions of Bub1 (which do not have a dimerization domain) under the *GALI* promoter because it led to strong signals on Western blots and had no effect on cell cycle progression (data not shown). We then prepared lysates from the cultures for co-IP. An anti-FLAG immunoprecipitate from an asynchronous culture (no benomyl and

nocodazole) contained a low level Bub1-13×Myc in addition to Bub1-3×FLAG, indicating that Bub1 can associate with itself and potentially form a dimer (**Figure 3-8A**). The low level of Bub1-13×Myc in the immunoprecipitates suggests that the interaction only involves a fraction of the total Bub1 in the cells. Arresting cells in metaphase by benomyl and nocodazole had no obvious effect on the level of Bub1-13×Myc in the anti-FLAG immunoprecipitate (**Figure 3-8A**), which shows that the Bub1-Bub1 interaction is not cell cycle regulated and is largely unaffected by activation of the spindle checkpoint. An anti-Myc immunoprecipitate from the same strain contained Bub1-3×FLAG as well as Bub1-13×Myc (**Figure 3-8A**), confirming that Bub1 proteins are able to interact with each other.

We noticed that the mobility of Bub1-13×Myc and Bub1-3×FLAG in the anti-FLAG immunoprecipitates was sometimes slower when compared to that in the total lysates (**Figure 3-8A**). This was not observed in the anti-Myc immunoprecipitates. Treating the anti-FLAG immunoprecipitates with phosphatase had no effect on the migration of the bands (data not shown), indicating that the change in mobility is not due to phosphorylation. We believe the shift is simply an artifact of co-IP experiments with anti-FLAG antibodies.

We then asked whether the Bub1-Bub1 interaction requires the presence of other checkpoint proteins. We introduced *P<sub>GALI</sub>-BUB1-3×FLAG* and *P<sub>GALI</sub>-BUB1-13×Myc* into diploid yeast strains with both copies of different spindle checkpoint genes deleted (*mad1Δ*, *mad2Δ*, *mad3Δ*, *bub1Δ*, and *bub3Δ*) and repeated the co-IP experiments. In all cases, both Bub1-3×FLAG and Bub1-13×Myc were found in the anti-FLAG immunoprecipitates, indicating that the interaction between two Bub1 proteins is independent of the presence of other components of the checkpoint (**Figure 3-8B**). Our results suggest that the Bub1-Bub1 interaction is constitutive

Protein(s) Expressed	Bub1-3xFLAG Bub1-13xMyc				Bub1-3xFLAG		Bub1-13xMyc		Bub1-3xFLAG Bub1-13xMyc	
	Total	IP	Total	IP	Total	IP	Total	IP	Total	IP
WB: Bub1-3xFLAG										
WB: Bub1-13xMyc										
	IP: anti-FLAG								IP: anti-Myc	

Proteins Expressed	Bub1-3xFLAG Bub1-13xMyc									
	<i>mad1Δ</i>		<i>mad2Δ</i>		<i>mad3Δ</i>		<i>bub1Δ</i>		<i>bub3Δ</i>	
	Total	IP	Total	IP	Total	IP	Total	IP	Total	IP
WB: Bub1-3xFLAG										
WB: Bub1-13xMyc										

IP: anti-FLAG

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and may act as an upstream component in the spindle checkpoint pathway.

## **Conclusions and Discussion**

We found that expressing the Bub1 dimer leads to metaphase arrest and spindle checkpoint activation in cells. Three different dimerization methods produced the same result, arguing that dimer formation, rather than a particular mechanism of dimerization, activates the checkpoint. The phenotype is independent of the kinase and Bub3-binding domains of Bub1 but requires the N-terminal kinetochore-targeting region. The metaphase arrest depends on the presence of most other checkpoint proteins, suggesting that the Bub1 dimer is acting upstream in the spindle checkpoint pathway. Chromosome bi-orientation is compromised in cells expressing Bub1-LacI, but the Bub1 dimer is able to arrest cells even in the absence of functional kinetochores, showing that the Bub1 dimer may activate the spindle checkpoint through multiple mechanisms. We found that Bub1 may form dimers during normal cell cycle independently of other checkpoint components, which suggests that the Bub1 dimer has functional roles in cells, such as initiating the spindle checkpoint.

The major finding from our experiments is that expressing the Bub1 dimer activates the spindle checkpoint. Overexpressing wild type Bub1 in budding yeast increases the rate of chromosome loss but has little effect on cell cycle progression [31], suggesting that the cell cycle arrest is induced by Bub1 dimerization rather than simply a high cellular level of Bub1 protein. We found that wild-type Bub1 may form dimers in cells, supporting the idea that the Bub1 dimer has a role in the spindle checkpoint. One possibility is that dimerization of Bub1 is important for its interaction with Mad1. During checkpoint activation, Mad1 dimerizes and recruits Mad2 to form a tetrameric 2:2 complex at the kinetochores [32-34]. It is not clear how Mad1 is localized

to the kinetochore. Bub1 associates with Mad1 in budding yeast, and the interaction only occurs when the spindle checkpoint is activated, suggesting that the binding is an important step in the checkpoint pathway [30]. A potential model is that Bub1 dimerizes at the kinetochore, and this dimer in turn binds a dimer of Mad1. This interaction may facilitate the localization and activation of Mad1, ultimately leading to the formation of Mad1-Mad2 complex at the kinetochore and spindle checkpoint activation.

When we overexpressed monomeric Bub1 in cells, only a fraction of them interacted with each other. One explanation is that Bub1 dimerizes strongly only when it is localized at the kinetochore. Since our results show that the Bub1 dimer can be found throughout the cell cycle, it will be necessary to regulate the interaction between Mad1 and Bub1 at the kinetochore if their association is important for checkpoint activation. Several mechanisms may help prevent the binding of the Bub1 dimer to Mad1 in the presence of proper microtubule attachments. Microtubule binding to the kinetochore may sterically block the interaction of Mad1 with Bub1 because the microtubule is in close proximity to Bub1 and leaves no room for Mad1 binding. It is also possible that microtubule binding leads to structural changes or post-translational modifications in Bub1 that prevent Mad1 binding. We speculate that when the artificial Bub1 dimer is overexpressed, a portion of the dimers can be found away from the kinetochore and thus escapes the inhibition. The dimers then directly lead to ectopic checkpoint activation, for example by inducing the production of Mad1-Mad2 complexes. If localization of the dimer away from the kinetochore leads to checkpoint activation, it would explain why the artificial Bub1 dimer activates the spindle checkpoint and arrests cells while the small quantities of Bub1 dimer found in cells expressing monomeric Bub1 fail to arrest the cell cycle. More experiments will be

needed to look at the interaction between Mad1 and the Bub1 dimer and determine whether the binding of Mad1 to Bub1 represents an important step in checkpoint activation.

## Materials and Methods

### Yeast Strains and Methods

All strains are derivatives of W303 (*ade2-1 his3-11,15 leu2-3,112 trp1-1 ura3-1 can1-100*), and are listed in **Table 3-1**. Strains were constructed using standard genetic techniques. All media were prepared using established recipes [35], and contain 2% wt/vol of the indicated sugar as the carbon source. To prepare media containing benomyl and nocodazole, DMSO stocks of methyl 1-(butylcarbamoyl)-2-benzimidazolecarbamate (benomyl) (Sigma-Aldrich, St. Louis, MO) and nocodazole (Sigma-Aldrich, St. Louis MO) were added to yeast extract and peptone (YEP) with 2% wt/vol glucose to a final concentration of 30µg/ml of each drug.

### Cell Cycle Analysis by Light Microscopy

To look at the effect of overexpressing different Bub1 constructs on cell cycle progression, cells were first grown to mid-log phase ( $10^7$  cells/ml) in YEP with 2% raffinose (wt/vol). Cells were then arrested in G<sub>1</sub> by adding 10µg/ml  $\alpha$ -factor (Bio-Synthesis, Lewisville, TX) and incubated for 2 hours at 30°C. Cells were washed four times to remove  $\alpha$ -factor and resuspended in YEP with either 2% glucose (wt/vol) or 2% galactose (wt/vol). After growing for 3 hours at 30°C, the cultures were briefly sonicated to separate cells that fail to dissociate completely after division and the percentage of large-budded cells in each sample was determined by light microscopy.

### Cell Cycle Analysis by Western Blots

To monitor cell cycle progression by Western blots, cells were grown and arrested in G<sub>1</sub> as described above, and released into the indicated media. 1µg/ml  $\alpha$ -factor was used for *bar1Δ*

strains. For experiments with *ndc10-1* strains, cells were grown to mid-log phase ( $10^7$  cells/ml) in YEP with 2% raffinose (wt/vol) at 25°C. Cells were then arrested in G<sub>1</sub> by adding 10µg/ml  $\alpha$ -factor and incubated for 2 hours at 25°C, and shifted to 37°C for 30 min to inactivate *ndc10-1*. Cells were washed four times to remove  $\alpha$ -factor and resuspended in the indicated media at 37°C. In all Western blot experiments, 10µg/ml  $\alpha$ -factor was added at 60 min after release from G<sub>1</sub> arrest to prevent cells from progressing into the next S phase.

For experiments with *mps1-as1* strains, DMSO (-Inhibitor) or 10µM of 1NM-PP1 (+Inhibitor) was added to the media after releasing the cells from G<sub>1</sub> arrest to inhibit the activity of Mps1-as1. For experiments with *ipl1-as5* strains, DMSO (-Inhibitor) or 50µM of 1NA-PP1 (+Inhibitor) was added to inhibit the activity of Ipl1-as5.

In all Western blot experiments, 1ml samples of the culture were collected at the indicated time points, and the cells were pelleted by centrifugation for 1 min at room temperature. The supernatant was removed, and cell pellets were stored at -80°C.

Cell pellets were lysed using a NaOH/ $\beta$ -mercaptoethanol-based protocol [36]. Proteins samples were loaded onto and separated in 10% Criterion Tris-HCl polyacrylamide gel (Bio-Rad, Hercules, CA). Proteins were transferred overnight to nitrocellulose (Whatman, Picataway, NJ). Western blotting for Myc-tagged securin were performed using anti-Myc 9E10 antibodies (Roche Applied Science, Indianapolis, IN) at a 1:500 dilution, and actin was detected with anti-actin antibodies (Abcam, Cambridge, MA) used at a 1:2000 dilution. Horseradish peroxidase-conjugated goat anti-mouse (Santa Cruz Biotechnology, Santa Cruz, CA) was used as the secondary antibody at a 1:2000 dilution. The secondary antibody was detected by SuperSignal West Dura Chemiluminescent Substrate (Thermo Fisher Scientific, Rockford, IL) and the blot was imaged with an AlphaImager (ProteinSimple, Santa Clara, CA).



## **Fluorescence Microscopy**

To look at cells arrested in metaphase by live-cell microscopy, cells were synchronized in G<sub>1</sub> with 1 µg/ml  $\alpha$ -factor and then subjected to a constant flow of media for 3 hours at room temperature using the ONIX microfluidic perfusion platform (CellASIC, Hayward, CA). Fluorescence microscopy was performed using Nikon Ti-E inverted microscope (Nikon, Melville, NY) equipped with a 60x objective (PlanApo, numerical aperture 1.4, oil), GFP filter (Chroma Technology, Bellow Falls, VT), and a CoolSNAP charge-coupled device camera (Photometrics, Tucson, AZ). Z-stacks of 25 sections were acquired using exposure times of 350 ms in Metamorph (Molecular Devices, Sunnyvale, CA). Z-stacks were combined into a single maximum intensity projection with ImageJ (NIH).

## **Co-Immunoprecipitation**

For immunoprecipitation experiments, cells were first grown to mid-log phase ( $10^7$  cells/ml) in 20ml of YEP with 2% raffinose (wt/vol) and resuspended in YEP with 2% galactose (wt/vol) for 3 hours. Cells were harvested by brief centrifugation and the pellets were immediately frozen with liquid nitrogen. Yeast extracts were made by vortexing frozen cell pellets in 200 µl of lysis buffer (100mM NaCl, 50mM Tris-HCl pH7.5, 10% glycerol, 50mM NaF, 50mM Na- $\beta$ -glycerolphosphate, 2mM EDTA, 0.1% Triton X-100, 1mM Na<sub>3</sub>VO<sub>4</sub>, 1mM PMSF, protease inhibitor cocktail) and around 100 µl of acid washed glass beads for three rounds of one minute, incubating on ice for one minute between each round. The resulting lysate was separated from the glass beads and centrifuged at 14,000 rpm for five minutes at 4°C to remove insoluble material. A portion of the lysate was mixed with an equal volume of 2X SDS sample buffer (Total).

Antibody was added to the remaining lysate at 1:50 dilution and incubated on ice for one hour. Samples were then transferred to 30 $\mu$ l of Protein A/G PLUS-agarose beads (Santa Cruz Biotechnology, Santa Cruz, CA) which had been equilibrated in lysis buffer. The beads were rotated at 4°C for one hour. The beads were then washed four times with lysis buffer and boiled in SDS sample buffer for ten minutes. The resulting samples (IP) were used for Western blotting as described above. Western blotting for Myc-tagged Bub1 was performed using anti-Myc 9E10 antibodies (Roche Applied Science, Indianapolis, IN) at a 1:500 dilution, and FLAG-tagged Bub1 was detected with anti-FLAG antibodies (Sigma-Aldrich, St. Louis, MO) used at a 1:2000 dilution.

Table 3-1. Strains used in this Chapter.

Strain Name	Genotype
DLY486	<i>MATa P<sub>GALI</sub>-BUB1-LacI@URA3</i>
DLY488	<i>MATa P<sub>GALI</sub>-BUB1@URA3</i>
DLY499	<i>MATa P<sub>GALI</sub>-BUB1-LacI@URA3 mad1Δ::HIS3</i>
DLY500	<i>MATa P<sub>GALI</sub>-BUB1-LacI@URA3 mad2Δ::Kan<sup>r</sup></i>
DLY501	<i>MATa P<sub>GALI</sub>-BUB1-LacI@URA3 mad3Δ::Kan<sup>r</sup></i>
DLY502	<i>MATa P<sub>GALI</sub>-BUB1-LacI@URA3 bub3Δ::Kan<sup>r</sup></i>
DLY508	<i>MATa P<sub>GALI</sub>-BUB1-LacI@URA3 PDS1::18xMYC::LEU2</i>
DLY545	<i>MATa P<sub>GALI</sub>-BUB1-LacI@URA3 bub1Δ::Kan<sup>r</sup></i>
DLY555	<i>MATa P<sub>GALI</sub>-BUB1-LacI(Δ1-60)@URA3</i>
DLY564	<i>MATa P<sub>GALI</sub>-bub1Δk-LacI@URA3</i>
DLY589	<i>MATa P<sub>GALI</sub>-BUB1-BUB1@URA3</i>
DLY958	<i>MATa P<sub>GALI</sub>-bub1ΔGLEBS-LacI@URA3</i>
DLY989	<i>MATa P<sub>GALI</sub>-BUB1-EEzip@URA3 P<sub>GALI</sub>-BUB1-RRzip@HIS3</i>
DLY1016	<i>MATa P<sub>GALI</sub>-bub1ΔGLEBS-LacI@URA3 mad1Δ::HIS3</i>
DLY1017	<i>MATa P<sub>GALI</sub>-bub1ΔGLEBS-LacI@URA3 mad2Δ::Kan<sup>r</sup></i>
DLY1018	<i>MATa P<sub>GALI</sub>-bub1ΔGLEBS-LacI@URA3 mad3Δ::Kan<sup>r</sup></i>
DLY1071	<i>MATa P<sub>GALI</sub>-bub1ΔGLEBS-LacI@URA3 bub1Δ::Kan<sup>r</sup></i>
DLY1090	<i>MATa P<sub>GALI</sub>-BUB1-LacI@URA3 P<sub>GALI</sub>-MAD3@HIS3</i>
DLY1091	<i>MATa P<sub>GALI</sub>-bub1ΔGLEBS-LacI@URA3 P<sub>GALI</sub>-MAD3@HIS3</i>
DLY1117	<i>MATa P<sub>GALI</sub>-BUB1-LacI@URA3 mps1Δ::Kan<sup>r</sup>::10xMYC-mps1-as1-TRP1</i> <i>PDS1::18xMYC::LEU2</i>
DLY1118	<i>MATa P<sub>GALI</sub>-BUB1-LacI@URA3 ipl1Δ::Kan<sup>r</sup>::ipl1-as5-LEU2 PDS1::18xMYC::LEU2</i> <i>bar1Δ</i>
DLY1119	<i>MATa P<sub>GALI</sub>-BUB1-LacI@URA3 ndc10-1 PDS1::18xMYC::LEU2</i>
DLY1131	<i>MATa P<sub>GALI</sub>-bub1ΔGLEBS-LacI@URA3 bub3Δ::Kan<sup>r</sup></i>

Table 3-1 (Continued). Strains used in this Chapter.

Strain Name	Genotype
DLY1141	<i>MATa P<sub>GALI</sub>-bub1ΔN-LacI@URA3</i>
DLY1142	<i>MATa P<sub>GALI</sub>-BUB1-LacI@ADE2 cdc20Δ:: P<sub>MET3</sub>-3×HA-CDC20-TRP1 P<sub>CUP1</sub>-GFP-LacI@HIS3 LacO256-URA3@CEN15 PDS1::18×MYC::LEU2 bar1Δ</i>
DLY1143	<i>MATa P<sub>GALI</sub>-bub1Δk-LacI @ADE2 cdc20Δ:: P<sub>MET3</sub>-3×HA-CDC20-TRP1 P<sub>CUP1</sub>-GFP-LacI@HIS3 LacO256-URA3@CEN15 PDS1::18×MYC::LEU2 bar1Δ</i>
DLY1162	<i>MATa/α P<sub>GALI</sub>-BUB1-3×FLAG @URA3/ P<sub>GALI</sub>-BUB1-13×MYC@URA3</i>
DLY1163	<i>MATa/α P<sub>GALI</sub>-BUB1-13×MYC@URA3/ura3-1</i>
DLY1179	<i>MATa P<sub>GALI</sub>-BUB1 @ADE2 cdc20Δ:: P<sub>MET3</sub>-3×HA-CDC20-TRP1 P<sub>CUP1</sub>-GFP-LacI@HIS3 LacO256-URA3@CEN15 PDS1::18×MYC::LEU2 bar1Δ</i>
DLY1190	<i>MATa/α P<sub>GALI</sub>-BUB1-3×FLAG @URA3/ P<sub>GALI</sub>-BUB1-13×MYC@URA3 mad1Δ::HIS3/ mad1Δ::HIS3</i>
DLY1191	<i>MATa/α P<sub>GALI</sub>-BUB1-3×FLAG @URA3/ P<sub>GALI</sub>-BUB1-13×MYC@URA3 mad2Δ::Kan<sup>r</sup>/ mad2Δ::Kan<sup>r</sup></i>
DLY1192	<i>MATa/α P<sub>GALI</sub>-BUB1-3×FLAG @URA3/ P<sub>GALI</sub>-BUB1-13×MYC@URA3 mad3Δ::Kan<sup>r</sup>/ mad3Δ::Kan<sup>r</sup></i>
DLY1193	<i>MATa/α P<sub>GALI</sub>-BUB1-3×FLAG @URA3/ P<sub>GALI</sub>-BUB1-13×MYC@URA3 bub1Δ::Kan<sup>r</sup>/ bub1Δ::Kan<sup>r</sup></i>
DLY1194	<i>MATa/α P<sub>GALI</sub>-BUB1-3×FLAG @URA3/ P<sub>GALI</sub>-BUB1-13×MYC@URA3 bub3Δ::Kan<sup>r</sup>/ bub3Δ::Kan<sup>r</sup></i>
DLY1197	<i>MATa/α P<sub>GALI</sub>-BUB1-3×FLAG @URA3/ura3-1</i>

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## **Chapter Four**

### **Mad2 and Mad3 Cooperate to Arrest Budding Yeast in Mitosis**



## Abstract

The spindle checkpoint ensures accurate chromosome transmission by delaying chromosome segregation until all chromosomes are correctly aligned on the mitotic spindle. The checkpoint is activated by kinetochores that are not attached to microtubules or are attached but not under tension and arrests cells at metaphase by inhibiting the anaphase-promoting complex (APC) and its co-activator Cdc20. Despite numerous studies, we still do not understand how the checkpoint proteins coordinate with each other to inhibit APC<sup>Cdc20</sup> activity.

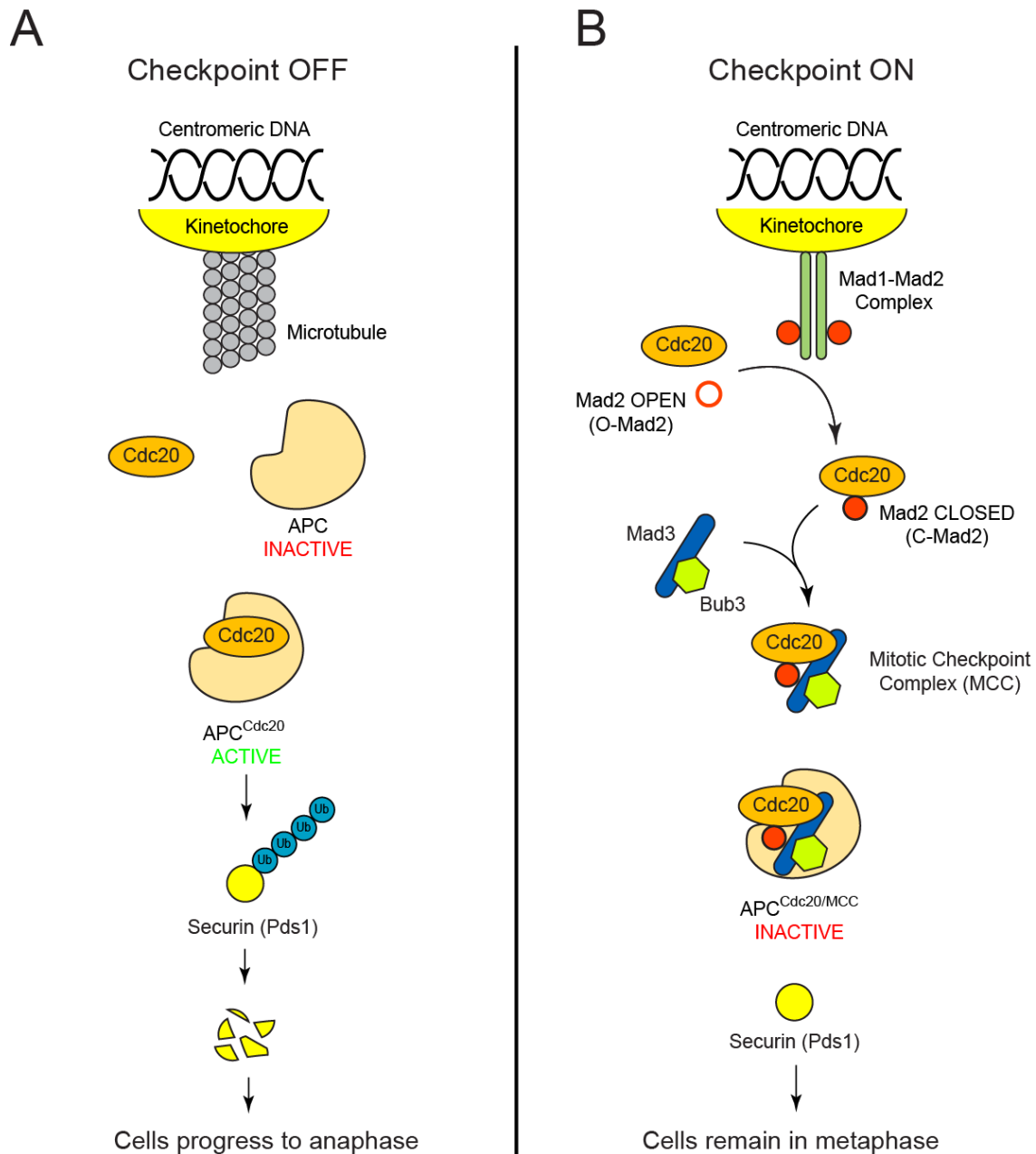
Inspired by the effect of expressing the Bub1 dimer, we constructed fusions of checkpoint proteins and expressed them in budding yeast to mimic possible protein interactions during checkpoint activation. We found that expression of a Mad2-Mad3 protein fusion or non-covalently linked Mad2 and Mad3, but not the overexpression of the two separate proteins, induces metaphase arrest that is independent of functional kinetochores or other checkpoint proteins. We further showed that artificially tethering Mad2 to Cdc20 also arrests cells in metaphase independently of other checkpoint components. Our results suggest that Mad3 is required for the stable binding of Mad2 to Cdc20 in vivo, which is sufficient to inhibit APC activity and is the most downstream event in spindle checkpoint activation.

This chapter is adapted from Lau, D.T.C., and Murray, A.W. (2012). Mad2 and Mad3 Cooperate to Arrest Budding Yeast in Mitosis. *Current Biology* 22, 180–190

## Introduction

Faithful segregation of genetic material during cell division is essential for the viability of all organisms. For each chromosome, DNA replication creates two identical copies, which are segregated from each other at mitosis. Segregation is directed by the kinetochore, a specialized multi-protein structure that assembles on centromeric DNA and binds to and moves along microtubules. Normal segregation depends on the two sister kinetochores attaching to microtubules from opposite spindle poles during mitosis. Eukaryotes use a control circuit called the spindle checkpoint to ensure accurate segregation. During unperturbed mitosis, an E3 ubiquitin ligase known as the anaphase-promoting complex (APC) and its co-activator Cdc20 triggers anaphase and chromosome segregation by catalyzing the ubiquitination and destruction of securin (Pds1 in budding yeast) (**Figure 4-1A**). The absence of microtubule attachment [1, 2] or the lack of tension at the kinetochore (because of chromosome failing to attach to opposite spindle poles) [3-5] activates the checkpoint, which arrests cells at the metaphase-to-anaphase transition by targeting APC and Cdc20 for inhibition (for reviews see [6, 7]). In the budding yeast, *Saccharomyces cerevisiae*, the key players of the spindle checkpoint include Mad1, Mad2, Mad3, Bub1, Bub3, Mps1, and Ipl1, all of which are highly conserved among eukaryotes [1, 2, 8, 9].

Although the checkpoint proteins have been studied extensively, we lack a molecular description of how events at the kinetochore are converted into inhibition of the APC. Several models have been described including the conformational change (Mad2-template) model [6], which proposes that Mad1-Mad2 complexes associate with kinetochores that lack microtubule attachments and recruit an “open” Mad2 conformer (O-Mad2), facilitating the formation of the “closed” Mad2 (C-Mad2)-Cdc20 complex (**Figure 4-1B**). Besides the recruitment of Mad1 and



**Figure 4-1.** A model for spindle checkpoint. **(A)** During mitosis, when all chromosomes are properly attached to microtubules, the anaphase-promoting complex (APC) and its co-activator Cdc20 polyubiquitinate different substrates such as securin (Pds1 in budding yeast), leading to its destruction and anaphase onset. **(B)** The spindle checkpoint is activated by the absence of microtubule attachment or the lack of tension at the kinetochore. Mad1-Mad2 complexes associate with unattached kinetochores and recruit the “open” Mad2 conformer (O-Mad2), facilitating the formation of a “closed” Mad2 (C-Mad2)-Cdc20 complex. The closed Mad2-Cdc20 complex associates with Mad3 and Bub3, which can be recruited to the kinetochores by interactions with Bub1 (not shown), to form the mitotic checkpoint complex (MCC). Inhibition of APC activity by the MCC arrests the cells in metaphase and gives the cells time to correct attachment errors at the kinetochores.

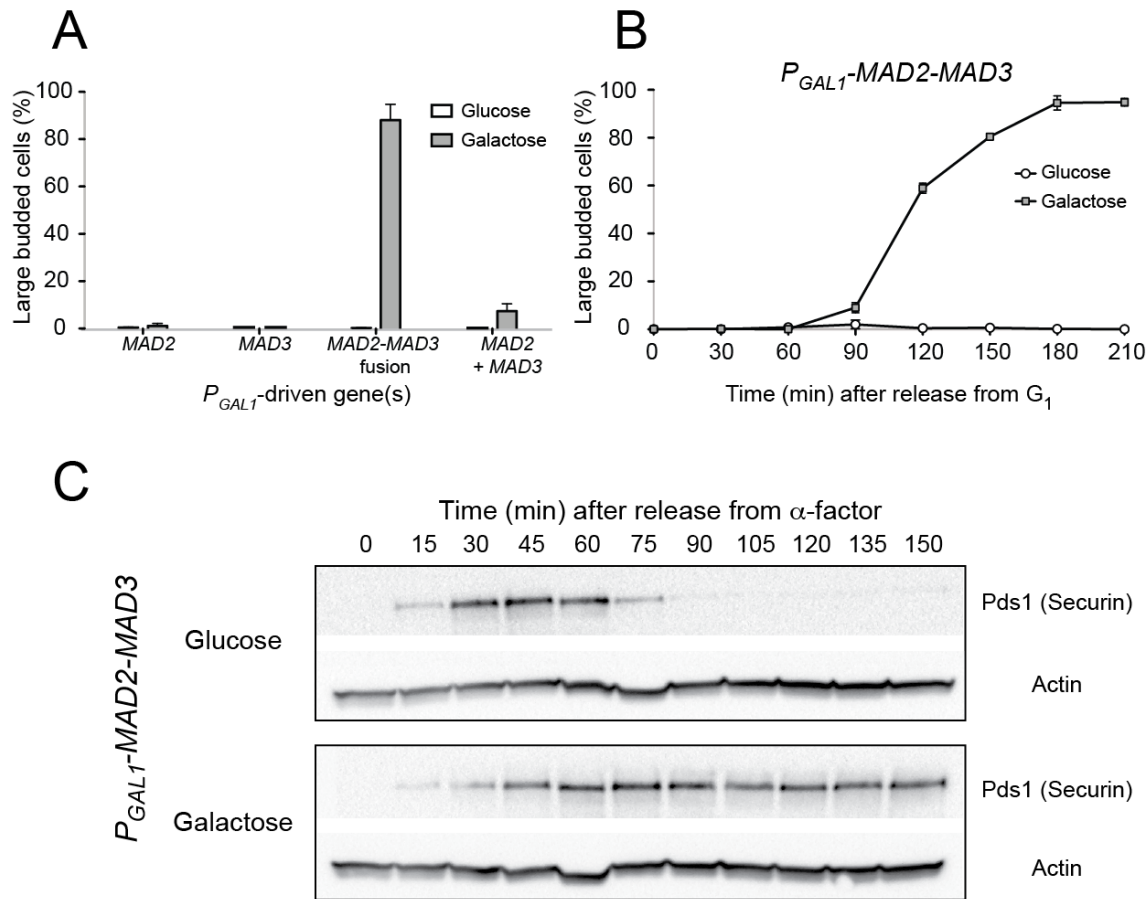
Mad2 to unattached kinetochores, experiments such as fluorescent protein localization and coimmunoprecipitation (co-IP) have shown that in budding yeast both Bub1 and Bub3 can associate with kinetochore [10] and Mad1 [11], while Mad3 can interact with both Mad2 and Bub3 [12]. This complicated network of interactions can potentially bring different checkpoint proteins together at the kinetochores in response to attachment errors and lead to formation of additional inhibitory complexes. One example is the mitotic checkpoint complex (MCC), which is proposed to consist of Mad2, Mad3, Bub3 and Cdc20 and has been shown to be a potent inhibitor of APC<sup>Cdc20</sup> [13, 14] (**Figure 4-1B**). Inhibition of APC activity arrests cells in metaphase and provides the cells a chance to correct the attachment errors at the kinetochores. The spindle checkpoint hence ensures that cells only progress through mitosis when all chromosomes are properly attached.

The initial studies that identified Cdc20 as the target of the spindle checkpoint showed that both Mad2 and Mad3 bind to Cdc20 [15]. We have investigated the consequences of this binding by manipulating the linkage between Mad2, Mad3, and Cdc20. Expressing physically-linked Mad2 and Mad3 induces a metaphase arrest that does not require functional kinetochores or other checkpoint proteins, indicating the Mad2-Mad3 fusion alone is sufficient to inhibit APC activity. We also show that tethering Mad2 directly to Cdc20 can lead to similar arrest that does not require Mad3 or other checkpoint components, supporting the idea that the Mad2-Mad3 fusion induces metaphase arrest by promoting an intimate association between Mad2 and Cdc20. Our results suggest that the most downstream event in spindle checkpoint activation is the cooperative binding of Mad2 and Mad3 to Cdc20.

## Results

### Expressing Physically-linked Mad2 and Mad3 Leads to Metaphase Arrest

Previous studies in budding yeast showed that both Mad2 and Mad3 are part of the MCC and associate with Cdc20 [12, 13, 15]. If Mad2 and Mad3 can both bind to Cdc20, checkpoint activation could strengthen the interaction between Mad2 and Mad3, making them bind more avidly to Cdc20 and arresting cells in mitosis. In this scenario, an engineered Mad2-Mad3 complex would artificially activate the spindle checkpoint. To test this idea, we asked if overexpressing different versions of Mad2 and Mad3 from the *GAL1* promoter could arrest wild-type yeast cells going through a synchronous cell cycle. We integrated the constructs for overexpression into the yeast genome without modifying the endogenous spindle checkpoint genes. The *GAL1* promoter is transcribed in the presence of galactose and inhibited by glucose. We arrested the cells in G<sub>1</sub> with  $\alpha$ -factor, then released them into media with either glucose or galactose, and looked at them three hours later. Cells that are cycling normally have no buds or buds that are clearly smaller than the mother cell, whereas cells that have trouble progressing through mitosis are enlarged and have distinctive large buds that approach the size of the mother cell. Overexpressing Mad2, Mad3, or both Mad2 and Mad3 together had little effect on the cells; more than 90% of the population continued to cycle when grown in galactose-containing medium (**Figure 4-2A**). In contrast, overexpressing a Mad2-Mad3 protein fusion, with the C terminus of Mad2 fused to the N terminus of Mad3 by an 8-amino acid linker, led to accumulation of large budded cells, a hallmark of metaphase arrest (**Figure 4-2A**). Expressing a fusion with Mad3 at the N terminus (Mad3-Mad2) produced similar result (data not shown). Cells arrested rapidly, as large budded cells started to accumulate at the first mitosis after inducing the Mad2-Mad3 fusion protein (**Figure 4-2B**). Overexpression of a Mad2-Mad3 fusion

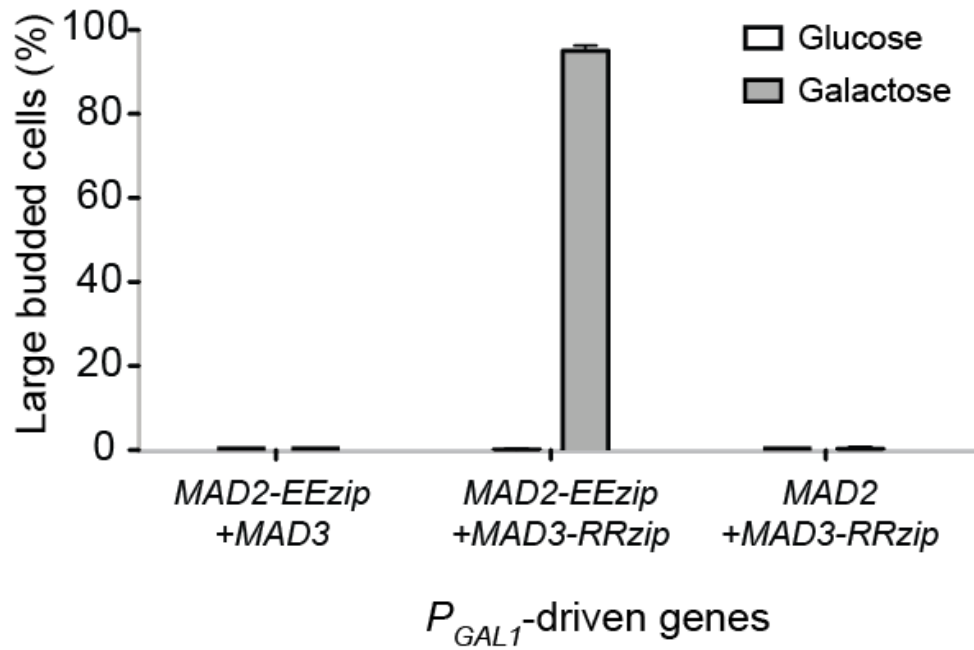


**Figure 4-2.** Overexpressing a Mad2-Mad3 protein fusion leads to metaphase arrest. **(A)** Cells with the indicated  $P_{GAL1}$ -driven genes were grown to mid-log phase, arrested in  $G_1$  with  $\alpha$ -factor, and were released into media with either glucose or galactose. After 3 hours of growth, the percentage of large budded cells was determined by light microscopy as a measure of metaphase arrest. Error bars represent the standard deviation of three independent trials. Two hundred cells were counted for each trial. **(B)**  $P_{GAL1}$ -MAD2-MAD3 cells were released from  $G_1$  arrest into glucose- or galactose-containing media. Samples were collected at the indicated time point and the percentage of large budded cells was determined by light microscopy. Error bars represent the standard deviation of three independent trials. Two hundred cells were counted at each time point for each trial. **(C)** Cell cycle progression of  $P_{GAL1}$ -MAD2-MAD3 cells was monitored by Western blots ( $n=3$ ), which detect securin (Pds1), a protein that is destroyed as cells enter anaphase. Cells were grown to mid-log phase and arrested in  $G_1$  with  $\alpha$ -factor, and were released into media with either glucose (top) or galactose (bottom). Western blots against Myc (to visualize Myc-tagged securin) or actin (loading control) were performed. When the cells were grown in glucose, securin level first increased and then dropped rapidly as cells progressed into anaphase. When the Mad2-Mad3 fusion was overexpressed in the presence of galactose, securin was stabilized, indicating that the cells were arrested in metaphase.

is therefore able to induce a strong metaphase arrest that does not occur when Mad2 and Mad3 are overexpressed as two independent proteins in the same cell.

We confirmed that expressing Mad2-Mad3 fusions prevents APC activation by using a biochemical marker for the exit into anaphase. The disappearance of securin (named Pds1 in budding yeast), a target of APC<sup>Cdc20</sup>, triggers the metaphase-to-anaphase transition and we followed the level of this protein by monitoring the level of epitope-tagged securin (Pds1-18xMyc) on Western Blots. When the Mad2-Mad3 fusion was not expressed (glucose), securin levels rose and fell after cells were released from G<sub>1</sub> arrest, indicating normal cell cycle progression (**Figure 4-2C, top**). Overexpressing the Mad2-Mad3 fusion (galactose) stabilized securin (**Figure 4-2C, bottom**).

We concluded that overexpressing the Mad2-Mad3 fusion inhibits APC and arrests cells in metaphase. To avoid the possibility that this arrest reflects some peculiarity of how Mad2 and Mad3 were fused together, we tested the effect of linking Mad2 and Mad3 using a different, non-covalent method. We fused Mad2 and Mad3 to engineered leucine zipper sequences (EEzip and RRzip) that allow the formation of stable heterodimers between two zippers bearing opposing charges [16]. When we expressed Mad2-Mad3 heterodimers (by releasing strains containing both *P<sub>GALI</sub>-MAD2-EEzip* and *P<sub>GALI</sub>-MAD3-RRzip* into galactose-containing medium), 90% of the cells arrested in metaphase (**Figure 4-3**). The phenotype is not observed when Mad2-EEzip was expressed with untagged Mad3 and vice versa, indicating that the arrest depends on interaction between Mad2 and Mad3 through the leucine zipper (**Figure 4-3**). The results confirm that overexpressing Mad2 and Mad3 can only induce metaphase arrest if the two proteins are held in close proximity.

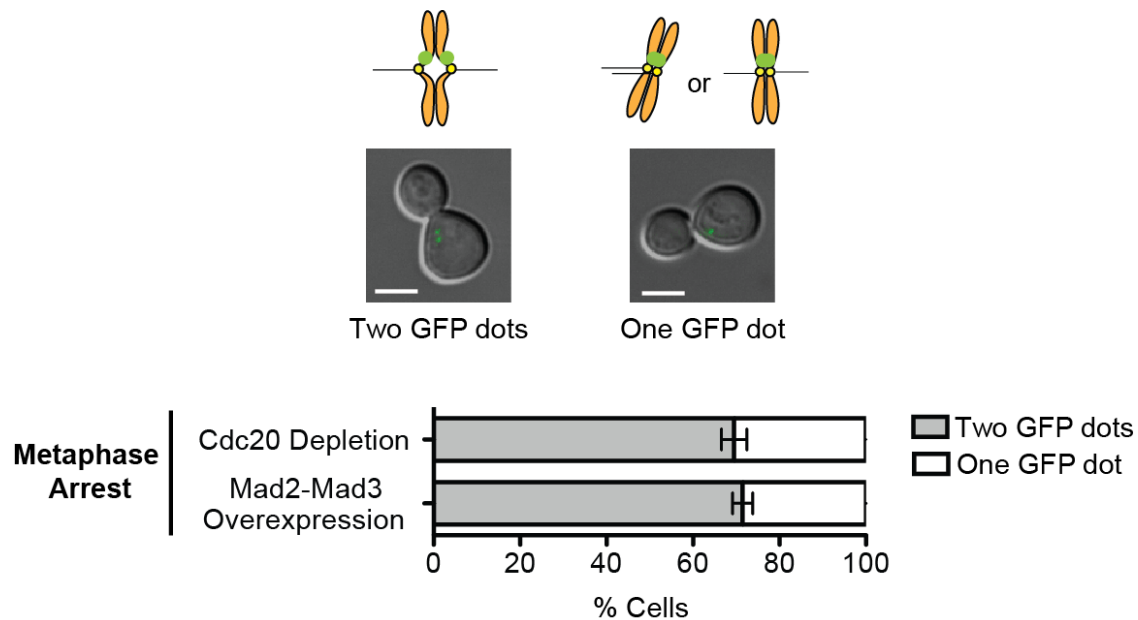


**Figure 4-3.** Overexpressing Mad2 and Mad3 linked by leucine zippers also induces metaphase arrest. Cells with the indicated  $P_{GAL1}$ -driven genes were released from  $G_1$  arrest into glucose- or galactose-containing media. The percentage of large budded cells was determined by light microscopy after 3 hours of growth. Error bars represent the standard deviation of three independent trials. Two hundred cells were counted for each trial.



## Mad2-Mad3 Fusion Does Not Affect Mitotic Spindle Structure

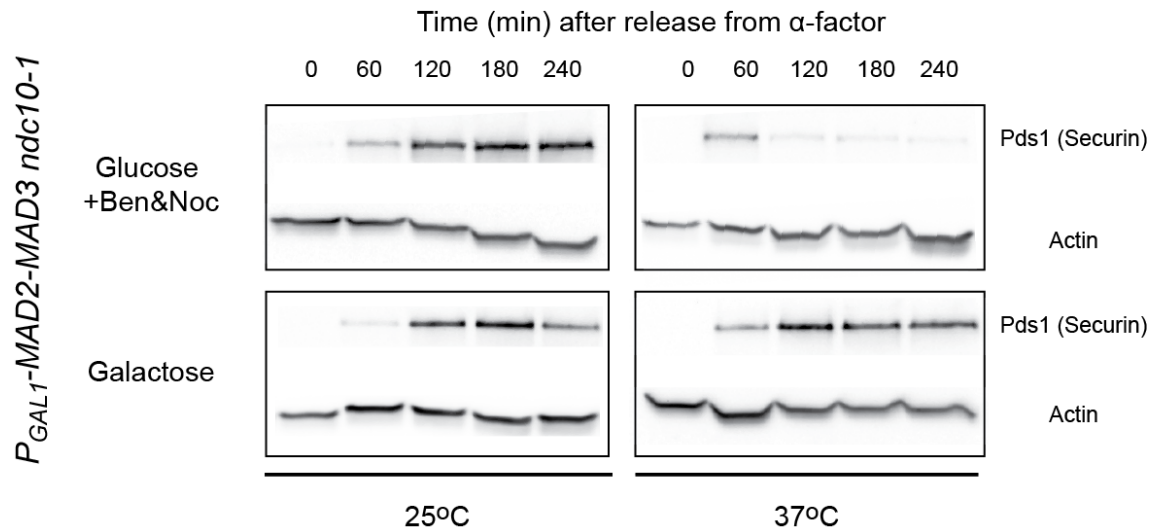
The Mad2-Mad3 fusion could lead to metaphase arrest in two ways: directly, by inhibiting APC<sup>Cdc20</sup> activity, or indirectly, by detaching microtubules from kinetochores, thus activating the spindle checkpoint. To determine the mode of checkpoint activation, we looked at chromosome bi-orientation in cells arrested in metaphase. A chromosome is said to bi-orient when the two sister kinetochores attach to microtubules from opposite spindle poles. The sister kinetochores can be pulled apart from each other by the spindle, and the separation, which can extend to about 0.5  $\mu\text{m}$ , can be visualized by expressing a GFP-Lac repressor fusion protein (GFP-LacI) and placing a lactose operator (LacO) array near the centromere [17-20]. Since bi-orientation is generated when chromosomes align correctly on the spindle, fewer chromosomes will bi-orient in cells arrested by Mad2-Mad3 overexpression if the fusion disrupts kinetochore-microtubule attachments or causes both sister kinetochores to attach to the same spindle pole. We integrated *P<sub>GALI</sub>-MAD2-MAD3* into a strain that expresses GFP-LacI, has a LacO array near the centromere of chromosome XV, and *CDC20* under the methionine-repressible *MET3* promoter (*P<sub>MET3</sub>-CDC20*). When the cells were grown in medium with glucose and methionine, Cdc20 was not expressed and the cells arrested in metaphase because they cannot activate the APC. Cdc20 depletion leads to metaphase arrest without disrupting the mitotic spindle, and around 70% of the cells arrested by this mechanism contained two GFP dots, indicating that chromosome XV bi-oriented (**Figure 4-4**). In about 30% of the cells, the separation between the two sister kinetochores is too small to allow the two LacO arrays to be resolved into two dots (**Figure 4-4**). The percentage of cells with two GFP dots was statistically indistinguishable when cells were arrested by overexpression of the Mad2-Mad3 fusion (by growing in medium with galactose and no methionine) (**Figure 4-4**). The results show that chromosomes bi-orient



**Figure 4-4.** Metaphase arrest by the Mad2-Mad3 fusion is independent of kinetochore-microtubule attachments. Chromosomes bi-orient in cells overexpressing the Mad2-Mad3 fusion. To achieve metaphase arrest, cells carrying *P<sub>GAL1</sub>-MAD2-MAD3*, GFP-LacI, a LacO array located near the centromere of chromosome XV, and *P<sub>MET3</sub>-CDC20* were released from G<sub>1</sub> arrest into either medium with glucose and methionine (Cdc20 depletion) or medium with galactose and no methionine (Mad2-Mad3 overexpression). Differential interference contrast (DIC) and GFP images of the cells were taken 3 hours after their release from G<sub>1</sub>. One or two GFP dots can be seen in cells arrested in metaphase. Cells have two GFP dots when chromosome XV bi-orient and the two sister kinetochores are separated by the spindle. Cells have one GFP dot when chromosome XV mono-orient (sister kinetochores attaching to the same spindle pole) or bi-orient but the two sister kinetochores are not pulled apart enough to allow resolution of two separate dots. Representative images of metaphase-arrested cells with one or two GFP dots are shown (Scale bar, 5 μm). Bar graph shows the percentage of cells with one or two GFP dots when they were arrested by Cdc20 depletion or overexpression of the Mad2-Mad3 fusion. Error bars represent the standard deviation of three independent trials. Two hundred cells were counted for each trial. Around 70% of cells arrested by Cdc20 depletion had two GFP dots. The percentage of cells that showed two GFP dots when the cells were arrested by the Mad2-Mad3 fusion is statistically indistinguishable ( $P=0.415$ ; two-tailed Student's *t* test). Cells therefore show normal chromosome bi-orientation when they are arrested in metaphase by the Mad2-Mad3 fusion, suggesting that the fusion does not disrupt kinetochore-microtubule connections.

normally during arrest induced by the Mad2-Mad3 fusion, suggesting that the fusion leads to metaphase arrest directly and does not disrupt normal spindle structure.

We next asked if the Mad2-Mad3 fusion could arrest cells that lacked kinetochores. The ability of microtubule poisons to activate the spindle checkpoint depends on the presence of functional kinetochores [21, 22]. Thus, demonstrating kinetochore-independent arrest would strengthen the conclusion that the Mad2-Mad3 fusion does not activate the checkpoint by disrupting kinetochore-microtubule connections. We looked at the phenotype of Mad2-Mad3 overexpression in the absence of functional kinetochores by using an *ndc10-1* strain. Ndc10 is a member of the CBF3 complex of the budding yeast kinetochore, which recognizes the centromeric DNA sequence and acts as the primary link between the chromosome and microtubule binding complexes of the kinetochore [23]. At the restrictive temperature (37°C), *ndc10-1* cells lack functional kinetochores and are therefore unable to activate the spindle checkpoint even in the presence of the microtubule depolymerizing drugs benomyl and nocodazole [21, 22]. If the Mad2-Mad3 fusion activates the checkpoint by disrupting microtubule attachment to the kinetochores, we should not observe metaphase arrest when the fusion is overexpressed in *ndc10-1* cells at 37°C. To test this prediction, we released *ndc10-1* cells carrying *P<sub>GAL1</sub>-MAD2-MAD3* from a G<sub>1</sub> arrest at 25°C or 37°C and monitored the level of securin. In cells that were released into glucose-containing medium (to inhibit expression of Mad2-Mad3) with benomyl and nocodazole (to depolymerize microtubules) at 37°C, securin levels rose and fell, showing that these cells failed to activate the spindle checkpoint (**Figure 4-5**) and confirming previous reports that kinetochores are required for normal checkpoint activation [21, 22]. In contrast, when these cells overexpressed the Mad2-Mad3 fusion because we released them into galactose-containing medium at 37°C, securin was stabilized (**Figure 4-5**).



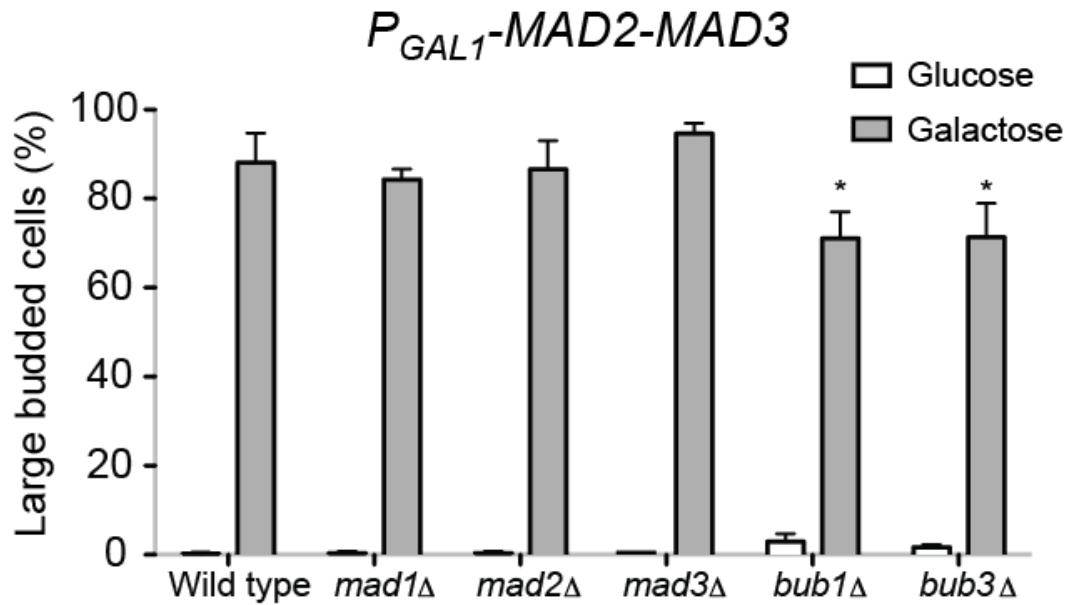
**Figure 4-5.** Metaphase arrest by the Mad2-Mad3 fusions does not require functional kinetochores. Cell cycle progression of cells with *P<sub>GAL1</sub>-MAD2-MAD3* and *ndc10-1* (a mutation that inactivates kinetochore at 37°C) was measured by Western blots (n=3). Cells were released from G<sub>1</sub> arrest into media with either glucose with benomyl and nocodazole (top) or galactose (bottom) at 25°C or 37°C. Western blots against Myc (to visualize Myc-tagged securin) or actin (loading control) were performed. Securin was stabilized in galactose-containing medium even at 37°C, indicating that the metaphase arrest is independent of functional kinetochores.

These results show that the Mad2-Mad3 fusion can arrest cells in metaphase even in the absence of functional kinetochores, supporting the idea that the fusion protein is acting downstream of the events at the kinetochores and directly inducing metaphase arrest without disrupting microtubule attachments to chromosomes.

### **Metaphase Arrest by Mad2-Mad3 Fusions Does Not Require Other Checkpoint Proteins**

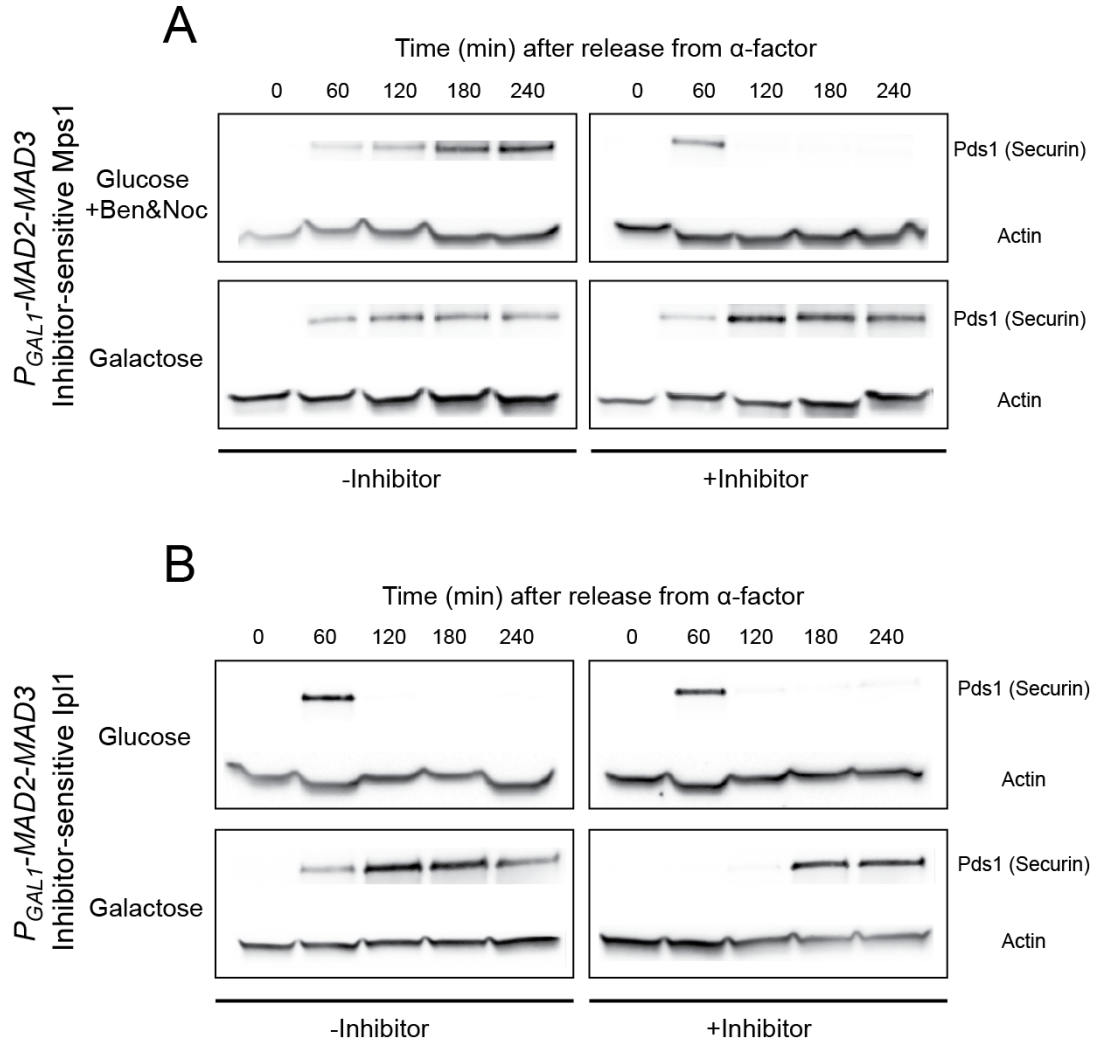
The Mad2-Mad3 fusion arrests cells in metaphase even when kinetochores are not assembled, showing that it bypasses at least one step of the normal spindle checkpoint and prompting us to ask whether other checkpoint components are similarly dispensable. We integrated the *P<sub>GALI</sub>-MAD2-MAD3* construct into yeast strains with different spindle checkpoint genes deleted (*mad1Δ*, *mad2Δ*, *mad3Δ*, *bub1Δ*, and *bub3Δ*) and tested the effect of expressing Mad2-Mad3 by releasing the cells from G<sub>1</sub> arrest into galactose-containing medium. In all cases, more than 70% of the population accumulated as large budded cells, indicating that the ability of the Mad2-Mad3 fusion to induce metaphase arrest does not require the presence of these checkpoint proteins (**Figure 4-6**). We noticed that *bub1Δ* and *bub3Δ* strains had a slightly lower percentage of large budded cells after 3 hours. The most likely explanation is that *bub1Δ* and *bub3Δ* cells grow more slowly and have a higher rate of death than wild type cells because of aneuploidy due to their high chromosome loss rate [24, 25].

Next we tested the requirement for two components of the spindle checkpoint, Mps1 and Ipl1, that have other essential functions. Mps1 is a kinase that is required for the spindle checkpoint, duplication of the spindle pole bodies (SPBs), and mitotic spindle assembly and function [26, 27]. Ipl1/Aurora B is another protein kinase, which is required to activate the spindle checkpoint in the absence of mechanical tension at the kinetochores [5, 28]; it is also



**Figure 4-6.** Metaphase arrest by Mad2-Mad3 fusions does not require other checkpoint components. Cells with *P<sub>GAL1</sub>-MAD2-MAD3* and deletion of the indicated checkpoint genes were released from G<sub>1</sub> arrest into glucose- or galactose-containing media. The percentage of large budded cells was determined by light microscopy after 3 hours of growth. Error bars represent the standard deviation of three independent trials. Two hundred cells were counted for each trial. Asterisks indicate significant difference from wild-type control (\**P* < 0.05; two-tailed Student's *t* test).

important for other processes such as spindle disassembly [29]. Since both Mps1 and Ipl1 are essential for cell viability, we used conditional alleles to inhibit their activities to ask whether they are required for the Mad2-Mad3 fusion to arrest cells. For Mps1, we used the analog-sensitive allele *mps1-as1* [27], which contains an enlarged ATP-binding pocket that makes this engineered kinase uniquely sensitive to a bulky protein kinase inhibitor [30]. In the absence of the inhibitor, such engineered kinases are functional, and in its presence, they are the only protein kinase whose activity is inhibited. We released *mps1-as1* cells carrying *P<sub>GALI</sub>-MAD2-MAD3* from G<sub>1</sub> arrest into media with or without the inhibitor (1NM-PP1, 1-(1, 1-dimethylethyl)-3-(1-naphthalenylmethyl)-1H-pyrazolo[3, 4-d]pyrimidin-4-amine) and monitored securin levels. Cells treated with the inhibitor that were grown in glucose-containing medium (to inhibit expression of Mad2-Mad3) with benomyl and nocodazole (to depolymerize microtubules) showed the normal rise and fall in securin (**Figure 4-7A**). This result confirmed that Mps1-as1 cannot function in the presence of the inhibitor and that cells normally cannot activate the spindle checkpoint in the absence of Mps1 activity. On the other hand, cells that were grown in galactose-containing medium (to express Mad2-Mad3) stabilized securin even in the presence of inhibitor (**Figure 4-7A**), indicating strong inhibition of APC activity by the Mad2-Mad3 fusion, despite the inactivation of Mps1. We performed similar experiments to test the requirement for Ipl1 by using the analog-sensitive allele, *ipl1-as5* [28]. We released *ipl1-as5* cells carrying *P<sub>GALI</sub>-MAD2-MAD3* from G<sub>1</sub> arrest into media with or without a slightly different inhibitor (1NA-PP1, 1-(1,1-dimethylethyl)-3-(1-naphthalenyl)-1H-pyrazolo[3, 4-d]pyrimidin-4-amine). Overexpressing the Mad2-Mad3 fusion stabilized securin even when Ipl1 was inactivated by adding the inhibitor (**Figure 4-7B**). We confirmed that Ipl1 activity in the strain is indeed sensitive to the inhibitor as they did not proliferate in the presence of 1NA-PP1 (data not shown).



**Figure 4-7.** Metaphase arrest by Mad2-Mad3 fusions does not require the checkpoint proteins Mps1 and Ipl1. **(A)** Cell cycle progression of cells with *P<sub>GAL1</sub>-MAD2-MAD3* and *mps1-as1* was monitored by Western blotting (n=3). Cells were released from G<sub>1</sub> arrest into media with either glucose with benomyl and nocodazole (top) or galactose (bottom), in the absence (-Inhibitor) or presence (+Inhibitor) of 1NM-PP1, an inhibitor of the analog-sensitive Mps1. Western blots against Myc or actin (loading control) were performed. Both securin and Mps1-as1 are tagged with Myc in the strain, but only the bands corresponding to Myc-tagged securin are shown in the figure. Securin was stabilized in galactose-containing medium even in the presence of inhibitor, indicating that Mps1 activity is not needed for the metaphase arrest. **(B)** The cell cycle progression of cells with *P<sub>GAL1</sub>-MAD2-MAD3* and *ipl1-as5* was measured by Western blots (n=3). Cells were released from G<sub>1</sub> arrest into media with either glucose (top) or galactose (bottom), in the absence (-Inhibitor) or presence (+Inhibitor) of 1NA-PP1, an inhibitor of the analog-sensitive Ipl1. Western blots against Myc (to visualize Myc-tagged securin) or actin (loading control) were performed. Securin was stabilized in galactose-containing medium in the presence of inhibitor, showing that Ipl1 activity is not needed for the metaphase arrest. The cause of the delay in accumulation of securin in cells grown in medium with galactose and the inhibitor is not known.



Our results thus show that both Mps1 and Ip11 activity are dispensable for metaphase arrest by the Mad2-Mad3 fusion.

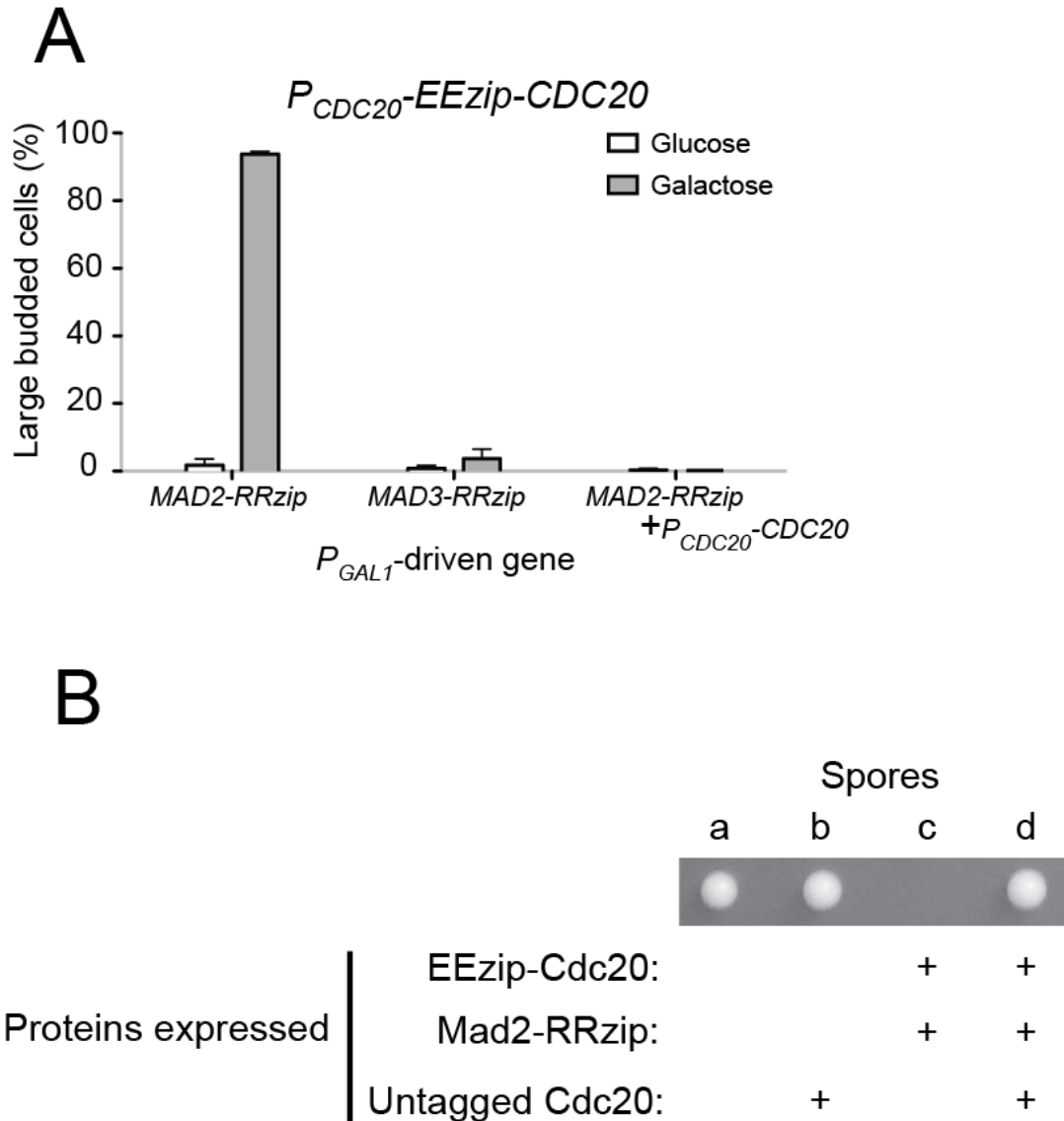
### **Directly Tethering Mad2 to Cdc20 Induces Metaphase Arrest**

Why does fusing Mad2 to Mad3 activate the checkpoint when the overexpression of the two separate proteins does not? One possibility is that each protein binds independently but weakly to Cdc20, whereas activation of the checkpoint requires stable binding of Mad2 or Mad3 to the Cdc20. In this scenario, the Mad2-Mad3 fusion would bind Cdc20 strongly since it would have a higher avidity for Cdc20 compared to Mad2 or Mad3 alone. Both the Mad2 and Mad3 region of the fusion protein can bind Cdc20, and either the Mad2-Cdc20 or Mad3-Cdc20 interactions would prevent the Mad2-Mad3 fusion from completely dissociating from Cdc20. Thus the fusion protein would bind Cdc20 tightly and inhibit APC<sup>Cdc20</sup> activity even in the absence of other checkpoint components. This model also explains how Mad2 and Mad3 could associate with Cdc20 in budding yeast throughout the cell cycle [12, 15] without activating the checkpoint until some signals from the checkpoint increased the strengths of the interactions between Mad2, Mad3, and Cdc20.

If the stable binding of Mad2-Mad3 to Cdc20 causes metaphase arrest, we should be able to induce a similar phenotype by artificially tethering Mad2 or Mad3 to Cdc20 to form a tight complex. To test this hypothesis, we investigated the effect of heterodimerizing Mad2 or Mad3 with Cdc20 using the engineered leucine zipper system [16]. We fused the endogenous *CDC20* to a negatively charged leucine zipper (making *P<sub>CDC20</sub>-EEzip-CDC20* as the only copy of *CDC20* in the cell) and integrated a construct with either *MAD2* or *MAD3* fused to a positively charged leucine zipper (*RRzip*) under the *GAL1* promoter (while leaving the endogenous *MAD2* and

*MAD3* loci intact). We released the cells from G<sub>1</sub> arrest into galactose-containing medium to overexpress the checkpoint fusion constructs, which would then be tethered to EEzip-Cdc20. In cells with *P<sub>CDC20</sub>-EEzip-CDC20* and *P<sub>GALI</sub>-MAD3-RRzip*, the majority of the population continued to cycle when they were grown in medium with galactose, showing that binding of Mad3 to Cdc20 alone was unable to stop the cell cycle (**Figure 4-8A**). In contrast, around 90% of cells expressing both *P<sub>CDC20</sub>-EEzip-CDC20* and *P<sub>GALI</sub>-MAD2-RRzip* arrested in mitosis (**Figure 4-8A**). The result shows that simply tethering Mad2 to Cdc20 leads to strong metaphase arrest. To confirm that the arrest is due to binding of Mad2 to Cdc20 via the leucine zippers, we performed the same experiment with a strain that expresses untagged Cdc20 in addition to EEzip-Cdc20 and Mad2-RRzip. In this case, the cells continued to cycle even in galactose-containing medium (**Figure 4-8A**), showing that the metaphase arrest is due to direct binding of Mad2 to Cdc20 and can be overcome by Cdc20 that is not tethered to Mad2. The normal cell cycle observed in cells with free (untagged) Cdc20 also suggests that tethering Mad2 to Cdc20 does not have any obvious detrimental effects besides inhibition of APC activity.

We then asked what would happen if Mad2-RRzip is expressed from the *MAD2* promoter instead of being overexpressed. Since the level of Mad2 in budding yeast exceeds that of Cdc20 [31], all Cdc20 should be tethered by Mad2 when both EEzip-Cdc20 and Mad2-RRzip are expressed from their endogenous promoters. We mated haploid cells carrying both *P<sub>CDC20</sub>-EEzip-CDC20* and *P<sub>CDC20</sub>-CDC20* (at the *URA3* locus) with cells carrying *P<sub>MAD2</sub>-MAD2-RRzip*. We then sporulated the diploids and dissected the tetrads to look at viability of the spores. Cells expressing both EEzip-Cdc20 and Mad2-RRzip failed to form visible colonies or only formed very small colonies (**Figure 4-8B and Table 4-1**). When the small colonies were examined microscopically, they were mostly made up of mitotically arrested cells (data not shown),



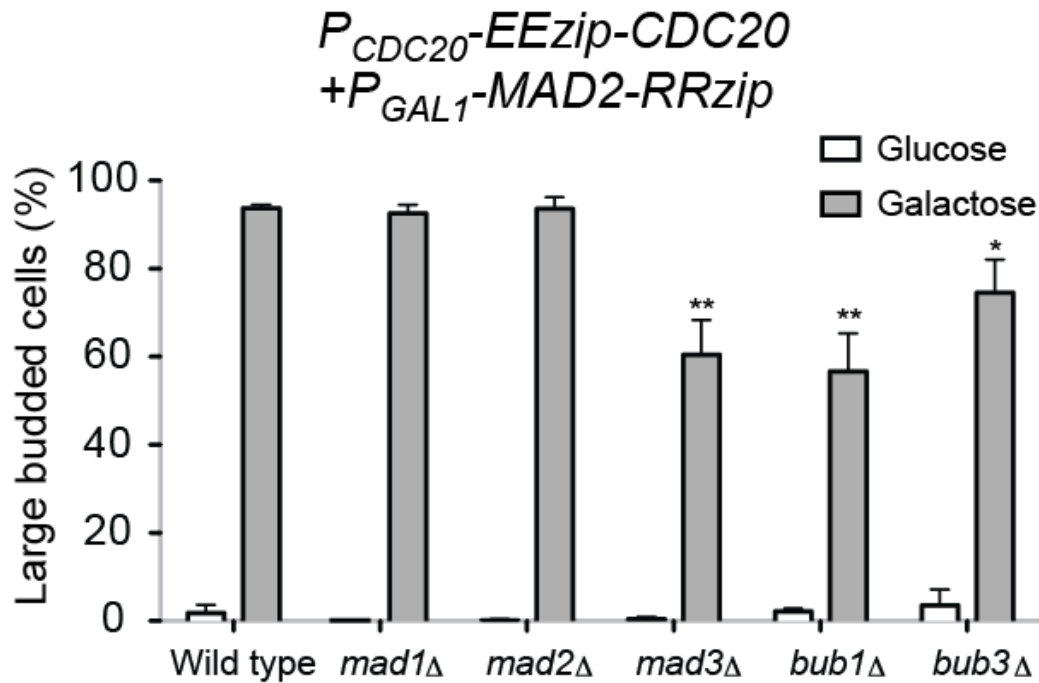
**Figure 4-8.** Tethering Mad2 directly to Cdc20 leads to metaphase arrest. **(A)** Cells with  $P_{CDC20}$ -*EEzip*-*CDC20* and the indicated  $P_{GAL1}$ -driven genes were released from G<sub>1</sub> arrest into glucose- or galactose-containing media. The percentage of large budded cells was determined by light microscopy after 3 hours of growth. Error bars represent the standard deviation of three independent trials. Two hundred cells were counted for each trial. Cells in the last column contain  $P_{CDC20}$ -*EEzip*-*CDC20*,  $P_{GAL1}$ -*MAD2*-RRzip, and an untagged copy of Cdc20 ( $P_{CDC20}$ -*CDC20*) and thus cannot be arrested in mitosis by expressing Mad2-RRzip. **(B)** Cells expressing EEzip-Cdc20 and Mad2-RRzip from the endogenous *CDC20* and *MAD2* promoters display growth defects that can be overcome by expressing untagged Cdc20. Diploids that are heterozygous for three manipulated genes,  $P_{CDC20}$ -*EEzip*-*CDC20*,  $P_{MAD2}$ -*MAD2*-RRzip, and  $P_{CDC20}$ -*CDC20*@*URA3*, were sporulated and a total of 15 tetrads were dissected (also see **Table 4-1**). Representative image of a tetrad on rich, glucose-containing plate after 2 days of growth at 30°C is shown. The “+” signs indicate proteins that are expressed based on the genotypes of each spore, which were determined by replica plating the tetrad onto dropout or drug plates. The genotype of spore c, which failed to form visible colonies, was inferred from the genotypes of other spores from the same tetrad.

Genotype	Colony size	
	Very Small/No colony	Regular
<i>P<sub>CDC20-EEzip</sub>-CDC20 P<sub>MAD2-MAD2-RRzip</sub></i>	7	0
<i>P<sub>CDC20-EEzip</sub>-CDC20 P<sub>MAD2-MAD2-RRzip</sub> P<sub>CDC20-</sub></i> <i>CDC20@URA3</i>	0	12
Number of tetrads dissected : 15		

**Table 4-1.** Colony size of spores with indicated genotypes from tetrad dissection (also see **Figure 4-8B**). The genotypes of the spores that failed to form colonies or formed very small colonies were inferred from the genotypes of the other spores assuming that all three heterozygously modified genes (*P<sub>CDC20-EEzip</sub>-CDC20*, *P<sub>MAD2-MAD2-RRzip</sub>*, and *P<sub>CDC20-CDC20@URA3</sub>*) showed Mendelian (2:2) segregation.

suggesting that the cells had trouble progressing through mitosis. On the other hand, cells that expressed untagged Cdc20 in addition to EEzip-Cdc20 and Mad2-RRzip showed normal growth (**Figure 4-8B and Table 4-1**). Our results show that the metaphase arrest seen in our earlier experiments is not simply due to a high, non-physiological level of the fusion proteins, since wild type expression of Mad2 is sufficient to arrest cells when Mad2 is constitutively tethered to Cdc20.

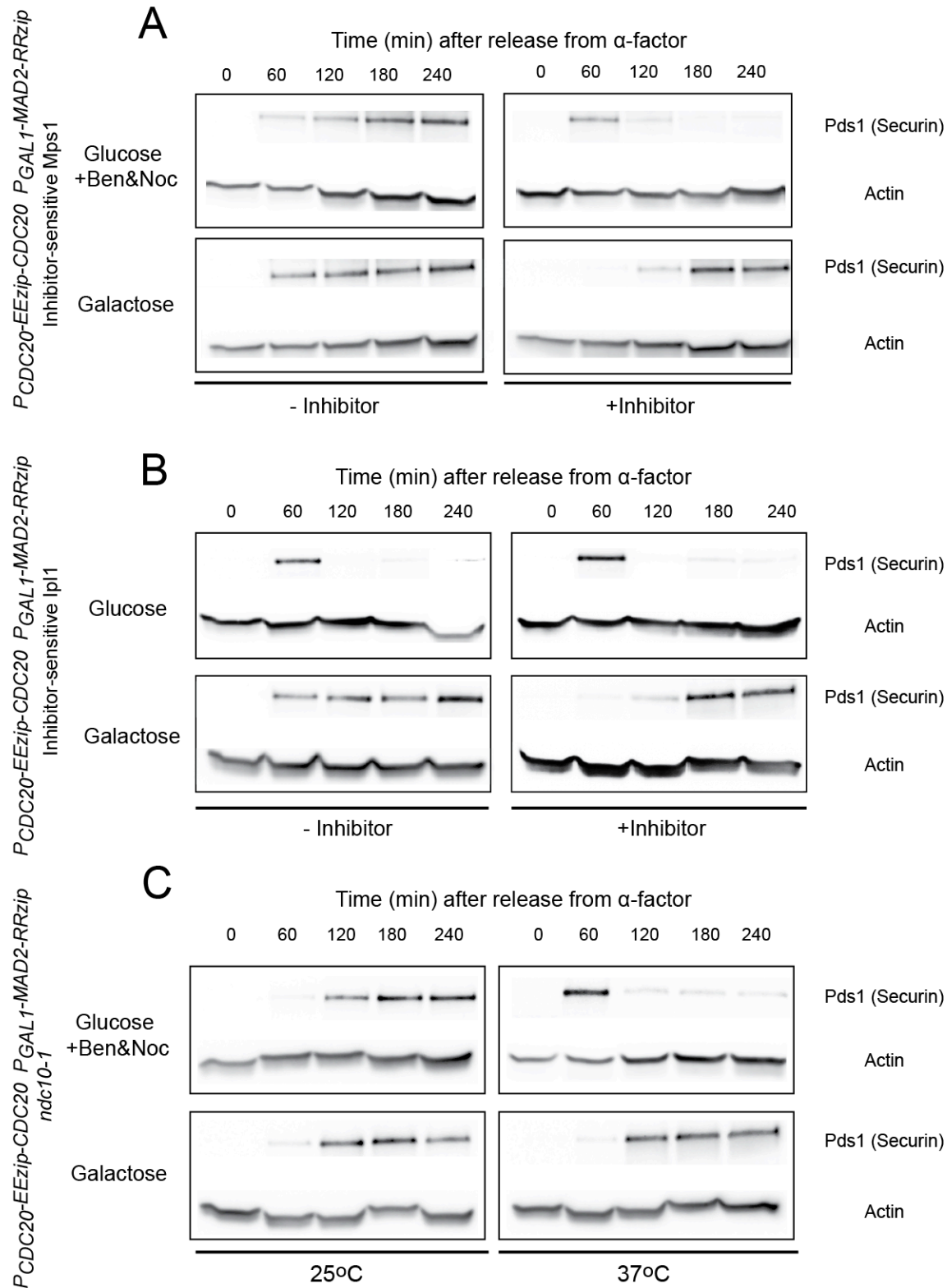
Since Cdc20 is the target of the spindle checkpoint, the stable binding of Mad2 to Cdc20 should be the last step in spindle checkpoint activation. If the hypothesis is correct, the arrest induced by tethering Mad2 to Cdc20 will not require other checkpoint components. To test this prediction, we introduced *P<sub>GAL1</sub>-MAD2-RRzip* and *P<sub>CDC20</sub>-EEzip-CDC20* into yeast strains with different checkpoint genes deleted (*mad1Δ*, *mad2Δ*, *mad3Δ*, *bub1Δ*, and *bub3Δ*). We looked at the effect of tethering Mad2 to Cdc20 by releasing these cells from G<sub>1</sub> arrest into galactose-containing medium. In all five checkpoint mutants, at least 55% of the population accumulated as large budded cells, showing that the metaphase arrest does not require the presence of these checkpoint proteins (**Figure 4-9**). We noted that the percentages of metaphase-arrested cells were significantly lower in *mad3Δ*, *bub1Δ*, and *bub3Δ* strains. The smaller number of large budded cells in *bub1Δ* and *bub3Δ* strains is again probably due to the growth defects exhibited by these strains. The weaker phenotype in *mad3Δ* cells, on the other hand, may suggest a role of Mad3 in strengthening the inhibition of Cdc20 by Mad2. The metaphase arrest also does not require Mps1, Ipl1, or functional kinetochores (**Figure 4-10**), further suggesting that tethering Mad2 to Cdc20 recapitulates a downstream event in checkpoint activation and leads to direct inhibition of APC.



**Figure 4-9.** The metaphase arrest produced by tethering Mad2 to Cdc20 does not require other checkpoint components. Cells with  $P_{CDC20}$ -*EEzip*-*CDC20*,  $P_{GAL1}$ -*MAD2*-*RRzip*, and deletion of the indicated checkpoint genes were released from G<sub>1</sub> arrest into glucose- or galactose-containing media. The percentage of large budded cells was determined by light microscopy after 3 hours of growth. Error bars represent the standard deviation of three independent trials. Two hundred cells were counted for each trial. Asterisks indicate significant difference from wild-type control (\* $P$  < 0.05 and \*\* $P$  < 0.01; two-tailed Student's  $t$  test).

**Figure 4-10.** Metaphase arrest induced by tethering Mad2 to Cdc20 does not require Mps1, Ipl1, or functional kinetochores. **(A)** Cell cycle progression of cells with *P<sub>CDC20</sub>-EEzip-CDC20*, *P<sub>GALI</sub>-MAD2-RRzip* and *mps1-as1* was monitored by Western blots (n=3). Cells were released from G<sub>1</sub> arrest into media with either glucose with benomyl and nocodazole (top) or galactose (bottom), in the absence (-Inhibitor) or presence (+Inhibitor) of 1NM-PP1, an inhibitor of the analog-sensitive Mps1. Western blots against Myc or actin (loading control) were performed. Securin was stabilized in galactose-containing medium in the presence of inhibitor, indicating that Mps1 activity is not required for the metaphase arrest. The cause of the delay in accumulation of securin in cells grown in medium with galactose and the inhibitor is not known. **(B)** The cell cycle progression of cells with *P<sub>CDC20</sub>-EEzip-CDC20*, *P<sub>GALI</sub>-MAD2-RRzip* and *ipl1-as5* was measured by Western blots (n=3). Cells were released from G<sub>1</sub> arrest into media with either glucose (top) or galactose (bottom), in the absence (-Inhibitor) or presence (+Inhibitor) of 1NA-PP1, an inhibitor of the analog-sensitive Ipl1. Western blots against Myc (to visualize Myc-tagged securin) or actin (loading control) were performed. Securin was stabilized in galactose-containing medium even in the presence of inhibitor, showing that Ipl1 activity is not needed for the metaphase arrest. The cause of the delay in accumulation of securin in cells grown in medium with galactose and the inhibitor is not known. **(C)** Cell cycle progression of cells with *P<sub>CDC20</sub>-EEzip-CDC20*, *P<sub>GALI</sub>-MAD2-RRzip* and *ndc10-1* was monitored by Western blots (n=3). Cells were released from G<sub>1</sub> arrest into media with either glucose with benomyl and nocodazole (top) or galactose (bottom) at 25°C or 37°C. Western blots against Myc (to visualize Myc-tagged securin) or actin (loading control) were performed. Securin was stabilized in galactose-containing medium at 37°C, showing that the metaphase arrest is independent of functional kinetochores.

Figure 4-10 (Continued).

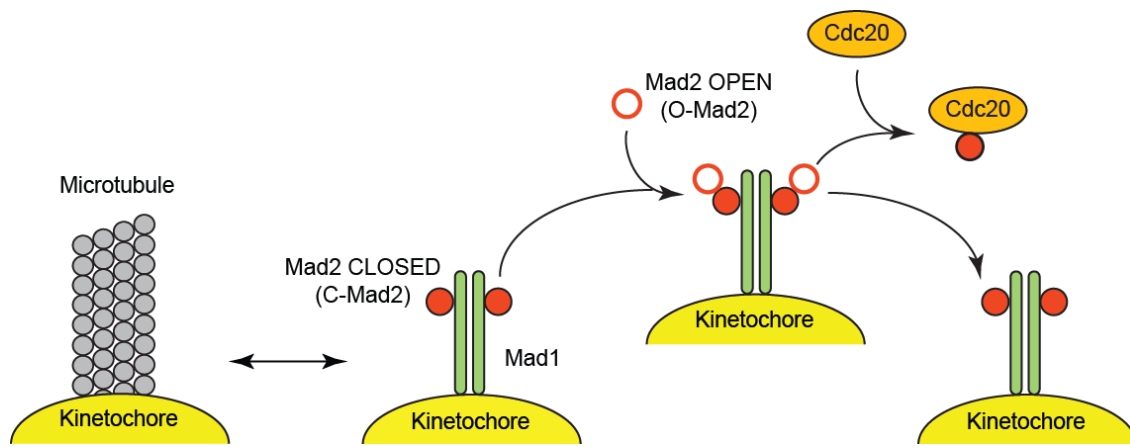




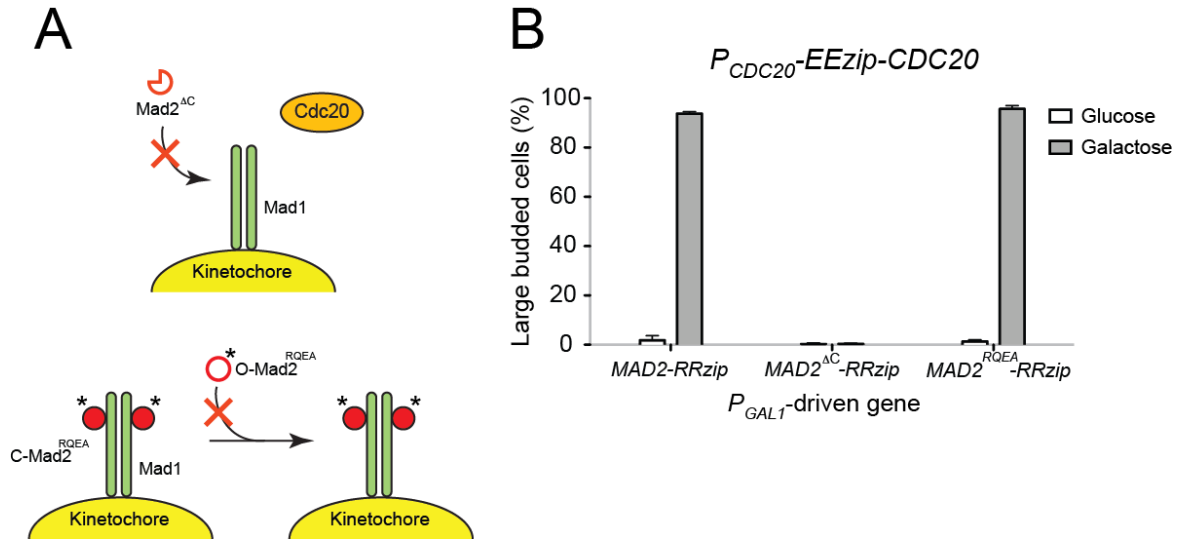
## The Phenotypes of Tethering Mad2 Mutants to Cdc20 Support the Mad2-template Model

The phenotypes we produced by tethering Mad2 to Cdc20 can be rationalized by the Mad2-template model. This model is based on the structures of different conformations of Mad2 [32-35], structural analysis of the Mad1-Mad2 complex [34], and imaging the dynamics of checkpoint proteins at the kinetochore [36-40]. In the model, Mad1 dimers associate with unattached kinetochores and bind Mad2 (**Figure 4-11**). This leads to the formation of Mad1-Mad2 complexes with Mad2 in the “closed” conformation (C-Mad2), which wraps around Mad1 or Cdc20. The complex in turn recruits a different conformer of Mad2, “open” Mad2 (O-Mad2), and facilitates its association with Cdc20 and conversion into closed Mad2. Mad2 can associate with Cdc20 throughout the budding yeast cell cycle [15], but this is insufficient to activate the checkpoint, likely because the interaction is too short-lived to allow the conformational change that is required to generate the closed Mad2-Cdc20 complex from open Mad2. The Mad1-Mad2 complex is therefore required to increase the rate of Mad2 conversion and produce the closed Mad2-Cdc20 complex [41]. The model predicts that if Mad2 can stably associate with Cdc20, it can eventually reach the closed conformation and inhibit  $APC^{Cdc20}$  even in the absence of attachment errors or other checkpoint proteins. Our observation that tethering Mad2 to Cdc20 can directly induce metaphase arrest is consistent with the model.

To further test the Mad2-template model, we tethered two previously studied Mad2 mutants to Cdc20. One mutant is Mad2 lacking its C-terminal 10 amino acid residues and was first characterized in HeLa cells [39]. Without this region, Mad2 is unable to close. The Mad2 mutant cannot activate the checkpoint as it fails to form a stable complex with Mad1 or to bind to and inhibit Cdc20 (**Figure 4-12A**). The corresponding Mad2 mutant in budding yeast ( $MAD2^{AC}$ ) also has no checkpoint function [42]. We integrated  $P_{GALI}-MAD2^{AC}-RRzip$  into cells expressing



**Figure 4-11.** The Mad2-template model. Mad1 dimers associate with unattached kinetochores and bind Mad2, converting them from “open” (O-Mad2) to “closed” (C-Mad2) conformation. The Mad1-Mad2 complexes at the kinetochores (the “templates”) then recruit additional open Mad2, allowing the formation of closed Mad2-Cdc20 complexes.



**Figure 4-12.** Metaphase arrest induced by tethering Mad2 mutants to Cdc20 supports the Mad2-template model. **(A)** The behavior of Mad2 mutants in the context of the Mad2-template model. (Top)  $\text{Mad2}^{\Delta\text{C}}$  lacks the C-terminal amino acid residues and cannot convert to the closed Mad2 conformation. It fails to activate the spindle checkpoint since it is unable to form a stable complex with Mad1 and to bind to and inhibit Cdc20. (Bottom) The double point mutant  $\text{Mad2}^{\text{RQEA}}$  carries the mutations Arg126-Glu and Gln127-Ala. The changes inhibit the binding between free Mad2 and closed conformation of Mad2 found in the Mad1-Mad2 complex, which inactivates the spindle checkpoint by preventing the formation of C-Mad2-Cdc20 complexes. The mutations also affect the interaction of Mad2 with BUBR1 (mammalian version of Mad3) and the formation of stable MCC. **(B)** Effects of tethering Mad2 mutants to Cdc20. Cells with  $P_{\text{CDC20}}\text{-EEzip-CDC20}$  and the indicated  $P_{\text{GAL1}}$ -driven genes were released from  $G_1$  arrest into glucose- or galactose-containing media. The percentage of large budded cells was determined by light microscopy after 3 hours of growth. Error bars represent the standard deviation of three independent trials. Two hundred cells were counted for each trial. Tethering the Mad2 mutant that can reach the closed conformation, but cannot induced conformational conversion in other Mad2 molecules ( $\text{Mad2}^{\text{RQEA}}$ ), does activate the checkpoint, but tethering the mutant that cannot achieve the closed conformation ( $\text{Mad2}^{\Delta\text{C}}$ ) does not.

EEzip-Cdc20. When the cells were grown in galactose-containing medium, the majority of the population continued to cycle (**Figure 4-12B**). Thus tethering Mad2<sup>ΔC</sup> to Cdc20 is unable to induce metaphase arrest, which supports the notion that only closed Mad2 can inhibit Cdc20.

Next we tested the Mad2 double point mutant Arg126-Glu/Gln127-Ala (*Mad2<sup>RQEA</sup>*). These two mutated residues have been conserved in eukaryotic evolution and are essential for the binding of soluble Mad2 to the closed conformation of Mad2 found in the Mad1-Mad2 complex [39, 42] (**Figure 4-12A**). Since this interaction is important for facilitating the formation of closed Mad2-Cdc20 complex, this Mad2 mutant does not have normal checkpoint function in either budding yeast [42] or HeLa cells [39]. These residues have also been shown to be important for the binding of Mad2 to BUBR1, the mammalian equivalent of Mad3, and the formation of MCC in vitro [43]. To test the effect of tethering Mad2<sup>RQEA</sup> to Cdc20, we integrated *P<sub>GALI</sub>-MAD2<sup>RQEA</sup>-RRzip* into cells expressing EEzip-Cdc20. When the cells were released into galactose-containing medium, 95% of them accumulated at the large-budded state (**Figure 4-12B**). The result suggests that when Mad2 is directly tethered to Cdc20, the interaction between closed and open Mad2 is dispensable since Mad2 can eventually close and inhibit Cdc20, which is again consistent with the Mad2-template model. Since human Mad2<sup>RQEA</sup> fails to bind to BUBR1, our experiment strengthens the conclusion that the binding between Mad2 and Mad3 and the formation of stable MCC is not necessary for the metaphase arrest induced by tethering Mad2 to Cdc20.

## **Conclusions and Discussion**

We showed that expressing a Mad2-Mad3 protein fusion arrests budding yeast in metaphase. The arrest does not require other checkpoint proteins and is not due to disruption of microtubule attachments to kinetochores. We obtained similar results by non-covalently linking Mad2 to Mad3 using leucine zippers, indicating that constitutive association between Mad2 and Mad3 is sufficient to prevent progression through mitosis. Finally, we showed that directly tethering Mad2 to Cdc20 also arrests cells in metaphase and that this arrest is independent of other checkpoint proteins. Our results support a model in which Mad2 and Mad3 are the most downstream components of the checkpoint pathway and cooperate to bind to Cdc20 and inhibit the APC.

### **Linking Mad2 and Mad3 Arrests Cells in the Absence of Spindle Damage**

A high level of Mad2 protein arrests cells in metaphase in a variety of organisms including *Xenopus* embryos [44, 45], fission yeast [46], and tissue culture cells [36, 39, 47]. The exact mechanism leading to the arrest likely differs between organisms; the arrest only requires Mad3 in fission yeast [48] and is independent of Mad1 in *Xenopus* embryos [44], but it requires Mad1 in tissue culture cells [47]. Our experiments show that in budding yeast, a high level of Mad2 alone is not enough to induce metaphase arrest, whereas expression of physically-linked Mad2 and Mad3 arrests cells independently of other checkpoint components. The results in different species may reflect differences in the interactions of Mad2 with other checkpoint proteins, the maximum expression of Mad2 that can be obtained, or the relative importance of Mad2 in checkpoint activation.

Mps1 overexpression in budding yeast can activate the spindle checkpoint without disrupting the mitotic spindle [49]. Unlike the metaphase arrest caused by linking Mad2 to Mad3, the effect of Mps1 overexpression depends on other checkpoint proteins; checkpoint mutants overexpressing Mps1 progress through mitosis without significant delay. Mps1 therefore likely represents an upstream activator that coordinates with other checkpoint proteins to activate the spindle checkpoint, while the Mad2-Mad3 fusion acts as a downstream effector that inhibits APC<sup>Cdc20</sup> even in the absence of other checkpoint proteins and functional kinetochores. Unlike many other checkpoint complexes previously identified in vivo [11-13], the Mad2-Mad3 fusion represents a minimal complex that can induce metaphase arrest independently of other known checkpoint components. Because we inactivated the other checkpoint proteins individually to test their requirements for arrest by Mad2-Mad3 fusions, we cannot rigorously exclude the possibility that two or more of them play a redundant role in helping the fusion to inhibit Cdc20, but we believe that Mad2-Mad3 is a direct inhibitor of APC<sup>Cdc20</sup> and functions downstream of events at the kinetochore.

### **Stable Binding of Mad2 to Cdc20 Can Lead to Metaphase Arrest**

We believe the Mad2-Mad3 fusion arrests cells because its high avidity for Cdc20 allows Mad2 to stay in close proximity to Cdc20 for long enough for Mad2 to adopt the closed conformation and inhibit Cdc20. Tethering Mad2 to Cdc20 using leucine zippers arrests cells in metaphase, supporting the hypothesis. Expressing both Mad2 and Cdc20 fused to leucine zippers from their endogenous promoters is sufficient to induce metaphase arrest in cells, showing that a physiological level of Mad2 can inhibit Cdc20 if the two proteins are forced to stably associate with each other. The inhibition by Mad2 is likely direct, as all other known checkpoint proteins

are dispensable for the arrest. We noticed that the metaphase arrest is weaker, but not absent, in *mad3Δ* cells. This result suggests that when Mad2 is stably associated with Cdc20, it can directly inhibit Cdc20 and recruit Mad3 to further strengthen the inhibition, and that while the recruitment of Mad3 potentiates Cdc20 inhibition, it is not essential to inactivate the APC in a significant fraction of cells. Consistent with this claim, most known checkpoint proteins, including Mad2, are necessary for the stable interaction between Cdc20 and Mad3 in budding yeast, whereas the binding of Mad2 to Cdc20 only requires Mad1 and Mps1 [12, 13, 15]. Our results support the hypothesis that Mad2 and Mad3 cooperate to bind to and inhibit Cdc20, and suggest that this event represents the last and essential step in spindle checkpoint activation. We believe that Mad3 has an auxiliary role and becomes dispensable when Mad2 can constitutively associate with Cdc20.

Tethering Mad3 to Cdc20 alone is unable to induce metaphase arrest in cells. We favor the interpretation that the main role of Mad3 is to promote the inhibition of Cdc20 by Mad2. In contrast, *in vitro* experiments have shown that BUBR1, the mammalian version of Mad3, could inhibit APC<sup>Cdc20</sup> alone and also act synergistically with Mad2 to repress APC activity [50, 51]. In budding yeast, Mad3 can also inhibit APC<sup>Cdc20</sup> *in vitro* in the absence of added Mad2 [Schuyler S, personal communication]. Several factors could account for the discrepancies between the *in vitro* data and our observations. The *in vitro* experiments, which contain reticulocyte lysate and APC purified from yeast or mammalian cells, may be contaminated by a low level of Mad2 that complicated the results. We also cannot exclude the possibility that inhibition of Cdc20 by Mad3 requires a specific orientation of the two proteins that cannot be achieved when they are tethered together by leucine zippers.

## Implications for the Mad2-template Model

The Mad2-template model is a prominent model that explains how the checkpoint proteins respond to events at the kinetochore and activate the spindle checkpoint. The model predicts that the requirements for kinetochores and other checkpoint proteins can be bypassed if Mad2 can stably associate with Cdc20, which is consistent with our result that tethering Mad2 to Cdc20 can directly induce metaphase arrest. The phenotypes observed when we tethered two known Mad2 mutants (Mad2<sup>ΔC</sup> and Mad2<sup>RQEA</sup>) to Cdc20 further support the Mad2-template model and strengthen our claim that the binding of Mad2 to Cdc20 is the most downstream event in checkpoint activation.

One extension from the Mad2-template model is that the closed Mad2-Cdc20 complex can recruit open Mad2 and trigger the production of additional closed Mad2-Cdc20, thereby amplifying the checkpoint signal [6]. When we tethered Mad2 to Cdc20 in cells that also express untagged Cdc20 (which cannot be tethered), the cells failed to arrest in metaphase, indicating that the Mad2-Cdc20 complex is unable to inhibit the untagged Cdc20. Amplification from closed Mad2-Cdc20 complexes is therefore unlikely to be a factor in further amplifying the checkpoint signal, and alternative mechanisms are required to ensure complete inhibition of APC<sup>Cdc20</sup> during normal checkpoint activation. Our results in cells with untagged Cdc20 also argue that tethering Mad2 to Cdc20 leads to direct inhibition of APC and does not have any obvious side effects as these cells progressed through the cell cycle normally.

Overall our results support the model that Mad2-Mad3 fusions and the association of Mad2 with Cdc20 inhibit APC activity by acting downstream of all other known checkpoint components. The two systems represent new ways for studying APC inhibition in vivo



independently of other checkpoint proteins and upstream kinetochore signals, which may allow us to better understand the molecular details of spindle checkpoint activation.

## Materials and Methods

### Yeast Strains and Methods

Strains used in this study are listed in **Table 4-2**. All strains are derivatives of W303 (*ade2-1 his3-11,15 leu2-3,112 trp1-1 ura3-1 can1-100*). Strains were constructed using standard genetic techniques. All media were prepared using established recipes [52], and contain 2% wt/vol of the indicated sugar as the carbon source. To prepare media containing benomyl and nocodazole, DMSO stocks of methyl 1-(butylcarbamoyl)-2-benzimidazolecarbamate (benomyl) (Sigma-Aldrich, St. Louis, MO) and nocodazole (Sigma-Aldrich, St. Louis MO) were added to yeast extract and peptone (YEP) with 2% wt/vol glucose to a final concentration of 30µg/ml of each drug. Diploid strains were sporulated in liquid culture by growing to saturation in YEP with 2% wt/vol glucose, diluting into YEP with 2% wt/vol potassium acetate for 12 hours at 30°C, washing with water, and resuspending in 2% potassium acetate at 25°C.

### Cell Cycle Analysis by Light Microscopy

To look at the effect of overexpressing different checkpoint constructs on cell cycle progression, cells were first grown to mid-log phase ( $10^7$  cells/ml) in YEP with 2% raffinose (wt/vol). Cells were then arrested in G<sub>1</sub> by adding 10µg/ml  $\alpha$ -factor (Bio-Synthesis, Lewisville, TX) and incubated for 2 hours at 30°C. Cells were washed four times to remove  $\alpha$ -factor and resuspended in YEP with either 2% glucose (wt/vol) or 2% galactose (wt/vol). After growing for 3 hours at 30°C, the cultures were briefly sonicated to separate cells that fail to dissociate completely after division and the percentage of large-budded cells in each sample was determined by light microscopy. For the time course experiment in Figure 2B, the cultures were

handled the same way except samples were taken every 30 min after releasing from G<sub>1</sub> arrest and counted.

### Cell Cycle Analysis by Western Blots

To monitor cell cycle progression by Western blots, cells were grown and arrested in G<sub>1</sub> as described above, and released into the indicated media. 1 µg/ml  $\alpha$ -factor was used for *bar1 $\Delta$*  strains. For experiments with *ndc10-1* strains, cells were grown to mid-log phase (10<sup>7</sup> cells/ml) in YEP with 2% raffinose (wt/vol) at 25°C. Cells were then arrested in G<sub>1</sub> by adding 10 µg/ml  $\alpha$ -factor and incubated for 2 hours at 25°C, and shifted to 37°C for 30 min to inactivate *ndc10-1*. Cells were washed four times to remove  $\alpha$ -factor and resuspended in the indicated media at 37°C. 10 µg/ml  $\alpha$ -factor was added at 60 min after release from G<sub>1</sub> arrest in all Western blot experiments to prevent cells from progressing into the next S phase.

For experiments with *mps1-as1* strains, DMSO (-Inhibitor) or 10 µM of 1NM-PP1 (+Inhibitor) was added to the media after releasing the cells from G<sub>1</sub> arrest to inhibit the activity of Mps1-as1. For experiments with *ipl1-as5* strains, DMSO (-Inhibitor) or 50 µM of 1NA-PP1 (+Inhibitor) was added to inhibit the activity of Ipl1-as5.

In all Western blot experiments, 1ml samples of the culture were collected at the indicated time points, and the cells were pelleted by centrifugation for 1 min at room temperature. The supernatant was removed, and cell pellets were stored at -80°C.

Cell pellets were lysed using a NaOH/ $\beta$ -mercaptoethanol-based protocol [53]. Proteins samples were loaded onto and separated in 10% Criterion Tris-HCl polyacrylamide gel (Bio-Rad, Hercules, CA). Proteins were transferred overnight to nitrocellulose (Whatman, Picataway, NJ). Western blotting for Myc-tagged securin were performed using anti-Myc 9E10 antibodies

(Roche Applied Science, Indianapolis, IN) at a 1:500 dilution, and actin was detected with anti-actin antibodies (Abcam, Cambridge, MA) used at a 1:2000 dilution. Horseradish peroxidase-conjugated goat anti-mouse (Santa Cruz Biotechnology, Santa Cruz, CA) was used as the secondary antibody at a 1:2000 dilution. The secondary antibody was detected by SuperSignal West Dura Chemiluminescent Substrate (Thermo Fisher Scientific, Rockford, IL) and the blot was imaged with an AlphaImager (ProteinSimple, Santa Clara, CA).

### **Fluorescence Microscopy**

To look at cells arrested in metaphase by the Mad2-Mad3 fusions using live-cell microscopy, cells were synchronized in G<sub>1</sub> with 1 µg/ml  $\alpha$ -factor and then subjected to a constant flow of indicated media for 3 hours at room temperature using the ONIX microfluidic perfusion platform (CellASIC, Hayward, CA). Fluorescence microscopy was performed using Nikon Ti-E inverted microscope (Nikon, Melville, NY) equipped with a 60x objective (PlanApo, numerical aperture 1.4, oil), GFP filter (Chroma Technology, Bellow Falls, VT), and a CoolSNAP charge-coupled device camera (Photometrics, Tucson, AZ). Z-stacks of 25 sections were acquired using exposure times of 350 ms in Metamorph (Molecular Devices, Sunnyvale, CA). Z-stacks were combined into a single maximum intensity projection with ImageJ (NIH).

Table 4-2. Strains used in this Chapter.

Strain Name	Genotype
DLY605	<i>MATa P<sub>GALI</sub>-MAD2@URA3</i>
DLY614	<i>MATa P<sub>GALI</sub>-MAD2-MAD3@URA3</i>
DLY663	<i>MATa P<sub>GALI</sub>-MAD2-MAD3@URA3 mad2Δ::Kan<sup>r</sup></i>
DLY664	<i>MATa P<sub>GALI</sub>-MAD2-MAD3@URA3 mad3Δ::Kan<sup>r</sup></i>
DLY665	<i>MATa P<sub>GALI</sub>-MAD2-MAD3@URA3 bub1Δ::Kan<sup>r</sup></i>
DLY685	<i>MATa P<sub>GALI</sub>-MAD2-MAD3@URA3 mad1Δ::HIS3</i>
DLY733	<i>MATa P<sub>GALI</sub>-MAD2@URA3 P<sub>GALI</sub>-MAD3@HIS3</i>
DLY752	<i>MATa P<sub>GALI</sub>-MAD2-MAD3@URA3 bub3Δ::Kan<sup>r</sup></i>
DLY885	<i>MATa cdc20Δ::P<sub>CDC20</sub>-EEzip-CDC20-Kan<sup>r</sup> P<sub>GALI</sub>-MAD2-RRzip@URA3</i>
DLY944	<i>MATa cdc20Δ::P<sub>CDC20</sub>-EEzip-CDC20-Kan<sup>r</sup> P<sub>GALI</sub>-MAD3-RRzip@URA3</i>
DLY945	<i>MATa cdc20Δ::P<sub>CDC20</sub>-EEzip-CDC20-Kan<sup>r</sup> P<sub>GALI</sub>-MAD2<sup>AC</sup>-RRzip@URA3</i>
DLY946	<i>MATa cdc20Δ::P<sub>CDC20</sub>-EEzip-CDC20-Kan<sup>r</sup> P<sub>GALI</sub>-MAD2<sup>RQEA</sup>-RRzip@URA3</i>
DLY953	<i>MATa cdc20Δ::P<sub>CDC20</sub>-EEzip-CDC20-Kan<sup>r</sup> P<sub>GALI</sub>-MAD2-RRzip@URA3 mad1Δ::HIS3</i>
DLY954	<i>MATa cdc20Δ::P<sub>CDC20</sub>-EEzip-CDC20-Kan<sup>r</sup> P<sub>GALI</sub>-MAD2-RRzip@URA3 mad2Δ::Kan<sup>r</sup></i>
DLY955	<i>MATa cdc20Δ::P<sub>CDC20</sub>-EEzip-CDC20-Kan<sup>r</sup> P<sub>GALI</sub>-MAD2-RRzip@URA3 bub3Δ::Kan<sup>r</sup></i>
DLY990	<i>MATa P<sub>GALI</sub>-MAD3@URA3</i>
DLY992	<i>MATa P<sub>GALI</sub>-MAD2-MAD3@URA3 PDS1::18×MYC::LEU2</i>
DLY996	<i>MATa cdc20Δ::P<sub>CDC20</sub>-EEzip-CDC20-Kan<sup>r</sup> P<sub>GALI</sub>-MAD2-RRzip@URA3 mad3Δ::LEU2</i>
DLY997	<i>MATa cdc20Δ::P<sub>CDC20</sub>-EEzip-CDC20-Kan<sup>r</sup> P<sub>GALI</sub>-MAD2-RRzip@URA3 bub1Δ::HIS3</i>
DLY1014	<i>MATa P<sub>GALI</sub>-MAD2-MAD3@URA3 mps1Δ::Kan<sup>r</sup>::10×MYC-mps1-as1-TRP1</i> <i>PDS1::18×MYC::LEU2</i>
DLY1036	<i>MATa P<sub>GALI</sub>-MAD2-MAD3@URA3 ndc10-1 PDS1::18×MYC::LEU2</i>
DLY1038	<i>MATa P<sub>GALI</sub>-MAD2-EEzip@HIS3 P<sub>GALI</sub>-MAD3-RRzip@URA3</i>

Table 4-2 (Continued). Strains used in this Chapter.

Strain Name	Genotype
DLY1061	<i>MATa P<sub>GALI</sub>-MAD2-MAD3@URA3 ipl1Δ::Kan<sup>r</sup>::ipl1-as5-LEU2 PDS1::18×MYC::LEU2 bar1Δ</i>
DLY1062	<i>MATa cdc20Δ::P<sub>CDC20</sub>-EEzip-CDC20-Kan<sup>r</sup> P<sub>GALI</sub>-MAD2-RRzip@URA3 mps1Δ::Kan<sup>r</sup>::10×MYC-mps1-as1-TRP1 PDS1::18×MYC::LEU2</i>
DLY1070	<i>MATa cdc20Δ::P<sub>CDC20</sub>-EEzip-CDC20-Kan<sup>r</sup> P<sub>GALI</sub>-MAD2-RRzip@URA3 ndc10-1 PDS1::18×MYC::LEU2</i>
DLY1074	<i>MATa P<sub>GALI</sub>-MAD2@HIS3 P<sub>GALI</sub>-MAD3-RRzip@URA3</i>
DLY1075	<i>MATa P<sub>GALI</sub>-MAD2-EEzip@HIS3 P<sub>GALI</sub>-MAD3@URA3</i>
DLY1076	<i>MATa cdc20Δ::P<sub>CDC20</sub>-EEzip-CDC20-Kan<sup>r</sup> P<sub>GALI</sub>-MAD2-RRzip@URA3 P<sub>CDC20</sub>-CDC20@HIS3</i>
DLY1077	<i>MATa cdc20Δ::P<sub>CDC20</sub>-EEzip-CDC20-Kan<sup>r</sup> P<sub>GALI</sub>-MAD2-RRzip@URA3 ipl1Δ::Kan<sup>r</sup>::ipl1-as5-LEU2 PDS1::18×MYC::LEU2</i>
DLY1079	<i>MATa/α P<sub>CDC20</sub>-EEzip-CDC20-Kan<sup>r</sup>/CDC20 P<sub>MAD2</sub>-MAD2-RRzip-His3MX6/MAD2 P<sub>CDC20</sub>-CDC20@URA3/ura3-1</i>
DLY1092	<i>MATa P<sub>GALI</sub>-MAD2-MAD3@ADE2 cdc20Δ::P<sub>MET3</sub>-3×HA-CDC20-TRP1 P<sub>CUP1</sub>-GFP-LacI@HIS3 LacO256-URA3@CEN15 PDS1::18×MYC::LEU2 bar1Δ</i>

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## **Chapter Five**

### **Conclusions, Discussion, and Future Directions**

## **Abstract**

Here we summarize the findings described in preceding chapters. We also discuss unresolved questions, such as the exact molecular details of metaphase arrest induced by the Bub1 dimer and the Mad2-Mad3 fusion, and propose experiments to address them in the future.

## Summary of Major Results

The spindle checkpoint is essential for accurate chromosome segregation during mitosis by inhibiting cell cycle progression until all chromosomes are properly lined up on the mitotic spindle. The kinetochore signals to the spindle checkpoint when attachment errors arise. The kinetochore may behave like a scaffold and recruit different spindle checkpoint components in the absence of proper microtubule attachment, thereby facilitating the assembly of diffusible inhibitory complexes and inducing cell cycle arrest (reviewed in Chapter One).

To determine whether localizing checkpoint proteins constitutes an important step in the checkpoint pathway, we created a system in budding yeast for recruiting checkpoint components fused to the lactose repressor (LacI) to an array of lactose operators (LacO) on the chromosome (Chapter Two). Recruiting the checkpoint protein Bub1 and a mutant version of Bub3 (*BUB3- $\Delta$ 117T*) to the chromosome appeared to activate the spindle checkpoint. We showed that the metaphase arrest is in fact caused by dimerization of Bub1 when it is fused to LacI rather than the recruitment of Bub1 to the chromosome (Chapter Three). The cell cycle arrest by the Bub1 dimer depends on the presence of other checkpoint proteins but not functional kinetochores. We found that a fraction of Bub1 may exist as a dimer during the normal cell cycle, which is consistent with the idea that the Bub1 dimer may be an upstream component in the spindle checkpoint pathway.

The fact that artificially dimerizing Bub1 is sufficient to activate the checkpoint led us to test the consequences of fusing checkpoint proteins to each other. We showed that fusing Mad2 and Mad3 arrests cells in metaphase independently of other checkpoint

proteins (Chapter Four). We believe that combining Mad2 and Mad3 arrests cells because the hybrid protein is able to bind tightly to Cdc20, the main target of the spindle checkpoint. We further demonstrated that artificially tethering Mad2 directly to Cdc20 also arrests cells and this arrest does not depend on any other checkpoint components. We concluded from our experiments that the stable binding of Mad2 and Mad3 to Cdc20 is sufficient to inhibit APC activity and represents the most downstream event in spindle checkpoint activation.

## **Discussion and Future Directions**

### **Localizing Spindle Checkpoint Proteins at the Kinetochores**

While our results provide some insights into the mechanism of spindle checkpoint activation, they raise many more unanswered questions. One of them is whether the model we started out to test (the kinetochore scaffold model) accurately describes how the spindle checkpoint is activated. Besides discovering the unexpected properties of Bub1-LacI, we found that recruiting other checkpoint protein fusions to a LacO array has no obvious effect on cell cycle progression. While the results may indicate that simply localizing checkpoint components to a DNA region is not sufficient to induce metaphase arrest, other factors may contribute to the lack of phenotype. First we cannot exclude the possibility that the functions of certain checkpoint proteins are compromised when they are fused to LacI. Second, the checkpoint proteins may have to be in specific orientations relative to each other when they are recruited to the kinetochore to activate the spindle checkpoint, and these orientations cannot be achieved when the same proteins are recruited to the LacO DNA via LacI. Third, the kinetochore may play an active role in

modifying the checkpoint components to allow checkpoint activation in addition to acting as a scaffold that binds checkpoint proteins. Simply recruiting the checkpoint proteins to a DNA region in the absence of kinetochore components will thus fail to mimic the actual events at the kinetochore. We attempted to address this issue by also expressing a protein fusion of the kinetochore protein Ask1 fused to LacI. We have previously shown that Ask1-LacI can bind to LacO and recruit other kinetochore components, forming a “synthetic kinetochore” at the LacO region that is able to perform many of the natural kinetochore functions [1]. Recruiting individual checkpoint proteins (besides Bub1-LacI) and a number of combinations of different checkpoint proteins to LacO repeats in the presence of the synthetic kinetochore again did not have any obvious effect (data not shown). It is possible that the synthetic kinetochore is missing certain kinetochore components that are involved in modifying the checkpoint proteins. Alternatively, the checkpoint proteins may need to have specific associations with the kinetochore, and simply localizing the synthetic kinetochore close to the checkpoint components is not sufficient to produce the necessary interactions.

One future direction is to determine the effect of localizing checkpoint proteins directly to the kinetochore, which is able to induce ectopic checkpoint activation in fission yeast [2] and human tissue culture cells [3]. We can either fuse checkpoint proteins directly to one of the kinetochore components, or use the engineered leucine zippers (EEzip and RRzip) to generate heterodimers between checkpoint proteins and components of the kinetochore. One advantage of using the leucine zippers is that we can systematically test all the possible pairings between checkpoint proteins and kinetochore components and determine whether localizing a particular checkpoint protein to a



specific region of the kinetochore complex is able to initiate the spindle checkpoint. The results may help us understand how the kinetochore interacts with the spindle checkpoint proteins to induce cell cycle arrest.

### **Roles of the Bub1 Dimer in the Spindle Checkpoint**

Another important question is how expression of the Bub1 dimer activates the spindle checkpoint. We hypothesize that dimerization of Bub1 is important for its interaction with Mad1. Bub1 may dimerize strongly only at the kinetochore, and the dimer in turn binds dimeric Mad1. The interaction may facilitate the localization and activation of the Mad1 dimer, ultimately leading to recruitment of Mad2 and formation of Mad1-Mad2 complexes. Bub1 associates with Mad1 in budding yeast when the spindle checkpoint is activated [4], supporting the idea that the interaction between Mad1 and Bub1 is important for the spindle checkpoint, but it is not known if Mad1 can interact with the Bub1 dimer. Additional experiments to look at the association between Mad1 and the Bub1 dimer will hopefully address the role of the Bub1 dimer in the spindle checkpoint.

If the interaction between the Bub1 dimer and Mad1 is important for activating the checkpoint, this association must be carefully regulated to prevent ectopic checkpoint activation since our results are consistent with the idea that at least a fraction of Bub1 is constitutively dimerized. We speculate that overexpression of the artificial Bub1 dimer arrests cells in metaphase because a fraction of the dimer escapes the regulation and thus activates the checkpoint, for example by stimulating the production of Mad1-Mad2 complexes. To verify this idea, it will be important to determine how the interaction

between Mad1 and the Bub1 dimer is normally controlled. Alternatively, there is no negative regulation on the Bub1 dimer and whether the checkpoint stops the cell depends on the balance between activation by the Bub1 dimer and inactivation by other mechanisms, such as dephosphorylation by protein phosphatase 1 (PP1) [5, 6]. When the kinetochores are properly attached, the dimer level is far too low to overcome the inactivating mechanisms. A huge increase in the dimer concentration by both overexpressing Bub1 and forcing it all to dimerize overwhelms the checkpoint inactivating mechanisms and thus arrests cells. It will be helpful to understand the localization and dynamics of the Bub1 dimer when it is overexpressed to determine whether it behaves differently from the endogenous Bub1 dimer.

The observation that expression of the Bub1 dimer interferes with chromosome bi-orientation is also worth following-up. Bub1 is important for phosphorylating histone 2A, which allows the recruitment of Sgo1 to the centromere to establish chromosome bi-orientation [7-9]. A high concentration of the Bub1 dimer may affect chromosome bi-orientation by sequestering the Bub1 targets away from the kinetochore. Determining the localization of the Bub1 dimer as well as the known downstream effectors such as Sgo1 will be an important step towards understanding the effect of the Bub1 dimer on chromosome bi-orientation. Furthermore, we can look at the dynamics of microtubule attachment in cells expressing Bub1-LacI to see whether the decrease in bi-oriented chromosomes is due to defect in fixing improper attachment or failure to stabilize correct microtubule binding.

## Metaphase Arrest By the Mad2-Mad3 Fusion

We do not yet have a complete picture of how the Mad2-Mad3 fusion arrests cells in metaphase. Our results suggest that the tight binding of the Mad2-Mad3 fusion to Cdc20 lead to inhibition of APC<sup>Cdc20</sup> and thus metaphase arrest. While the Mad2-Mad3 fusion is indeed able to bind Cdc20 (described in Appendix Two), additional experiments are needed to determine the exact nature of this interaction. It will also be interesting to address the molecular details of the inhibition of APC<sup>Cdc20</sup> activity by the Mad2-Mad3 fusion. One aspect we can look at is the stability of Cdc20 during metaphase arrest. Normally when the spindle checkpoint is activated, the half-life of Cdc20 is reduced and results in lower Cdc20 protein levels, which is important for preventing premature APC activation [10, 11]. In contrast, when cells are arrested in metaphase by Mad2-Mad3 expression, the protein level of Cdc20 in the cells remains stable (described in Appendix Two). We speculate that the difference in Cdc20 stability is due to the distinct roles of Mad2 and Mad3 in inhibiting APC<sup>Cdc20</sup>. Mad2 binds to the N-terminus of Cdc20 and interferes with the interaction between APC and the C-box of Cdc20, thereby inhibits activation of APC [12, 13]. On the other hand, Mad3 prevents Cdc20 from binding to its substrates, for example by occupying the KEN box-binding site of Cdc20 [13-16]. When the spindle checkpoint is activated, Cdc20 interacts with both Mad2 and Mad3, and the interactions are likely very dynamic. During instances when Cdc20 associates with Mad3 but not Mad2, Cdc20 can still bind APC through its C box, even though Cdc20 cannot bind to APC substrates and target them for ubiquitination. In the absence of these substrates, the APC will ubiquitinate Cdc20 and target its destruction. This accounts for the increased degradation of Cdc20 during checkpoint activation. When the Mad2-Mad3

fusion binds Cdc20, both Mad2 and Mad3 will associate with Cdc20 at the same time. The binding of Mad2 prevents the interaction between Cdc20 and APC and therefore stabilizes Cdc20, thus explaining why the level of Cdc20 stays relatively constant in cells arrested by Mad2-Mad3 expression. One potential way to test this model is to carry out in vitro APC assays to carefully determine the effect of the Mad2-Mad3 fusion on the binding of Cdc20 to APC as well as APC activity. In addition, we can introduce Mad1 and the Bub1 dimer to the system to further mimic checkpoint activation in vitro. This may ultimately allow us to biochemically determine how the reactions that start at the kinetochore lead to changes in the ability of Mad2 and Mad3 to bind to Cdc20 and inhibit the APC.

We found that in addition to Mad2-Mad3, expression of two other protein fusions, Bub1-Mad2 and Bub1-Bub3, also activates the spindle checkpoint (discussed in Appendix One). Preliminary experiments showed that the metaphase arrest by these fusions require the presence of other checkpoint components, which suggest that the protein fusions are acting upstream in the checkpoint pathway similar to the Bub1 dimer. It will be interesting to further characterize their effects, and the findings from these experiments may provide additional insights into the interactions among checkpoint components that are important for checkpoint activation.

### **Closing Remarks**

Overall our results demonstrate that manipulating the associations between checkpoint proteins can have a huge impact on spindle checkpoint activation. Cells thus have to carefully control the interactions among the spindle checkpoint components to

prevent undesired cell cycle arrest. How this is achieved is still poorly understood. Future work on this area may get us a little bit closer to the holy grail of the spindle checkpoint: understanding how events at a single kinetochore can generate a molecular signal that is potent enough to completely inhibit APC activity and arrest cells in mitosis.

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## **Appendix One**

### **Spindle Checkpoint Activation by Checkpoint Protein Fusions**

#### **Abstract**

We tested the effect of expressing different checkpoint protein fusions on cell cycle progression. In Chapter Four we discussed our finding that expression of a Mad2-Mad3 fusion is sufficient to arrest cells in metaphase. Here we describe two other protein fusions, Bub1-Mad2 and Bub1-Bub3, which can also induce cell cycle arrest when overexpressed. Both of these fusions depend on other checkpoint components to activate the checkpoint, suggesting that they may mimic protein interactions that are important for upstream events in the spindle checkpoint pathway.



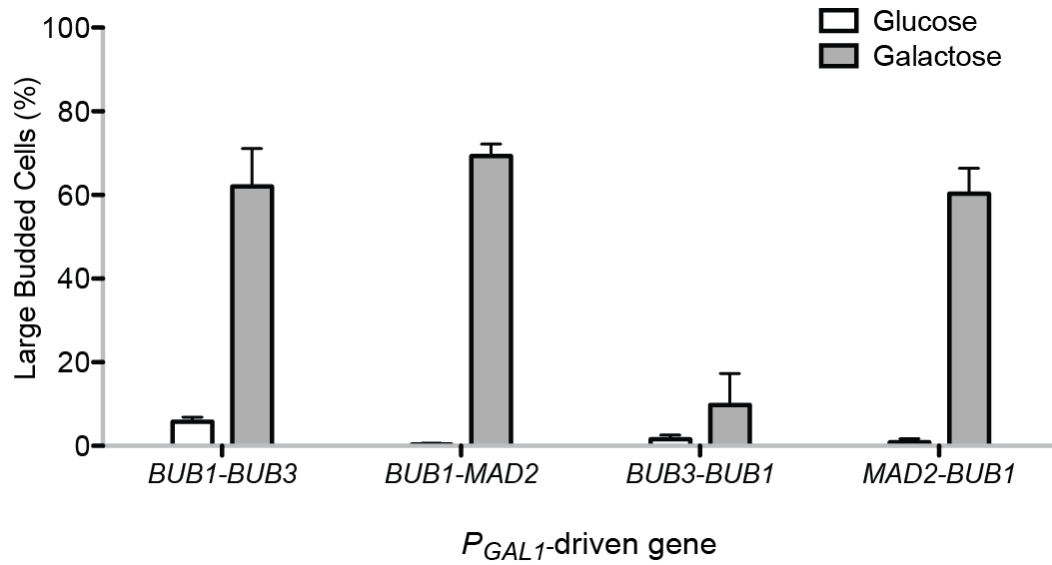
## Introduction

The ability of the Bub1 dimer to activate the spindle checkpoint (described in Chapter Three) motivated us to look at the effect of fusing different checkpoint proteins together. We found that expression of a Mad2-Mad3 protein fusion is sufficient to arrest cells in metaphase independently of other checkpoint components (discussed in Chapter Four), which is likely caused by the tight binding of the fusion to Cdc20.

In this section we summarize our observations that expression of two other protein fusions, Bub1-Mad2 and Bub1-Bub3, can also induce metaphase arrest in cells. Unlike Mad2-Mad3, these fusions require other checkpoint components in order to activate the checkpoint. The protein fusions may thus recapitulate interactions that are important for initiating the spindle checkpoint pathway.

## Results

To determine the effect of fusing different checkpoint proteins, we tested the consequences of expressing different protein fusions from the *GALI* promoter in wild-type yeast cells going through a synchronous cell cycle. We arrested the cells in G<sub>1</sub> with  $\alpha$ -factor, then released them into media with either glucose or galactose, and looked at them three hours later. Expression of Bub1-Bub3 and Bub1-Mad2, with the C-terminus of Bub1 fused to the N-terminus of either Bub3 or Mad2, led to accumulation of large budded cells and metaphase arrest (**Figure A1-1**). We also looked at the phenotype of expressing fusions of the checkpoint proteins at the opposite orientation (i.e. the C-terminus of Mad2 or Bub3 fused to the N-terminus of Bub1). Expressing a Mad2-Bub1 fusion still arrested the majority of the cells in metaphase, whereas expression of Bub3-



**Figure A1-1.** Expressing Bub1-Bub3 or Bub1-Mad2 protein fusions arrests cells in metaphase. Cells with the indicated  $P_{GAL1}$ -driven genes were grown to mid-log phase, arrested in  $G_1$  with  $\alpha$ -factor, and were released into media with either glucose or galactose. After 3 hours of growth, the percentage of large budded cells was determined by light microscopy as a measure of metaphase arrest. Error bars represent the standard deviation of three independent trials. Two hundred cells were counted for each trial.

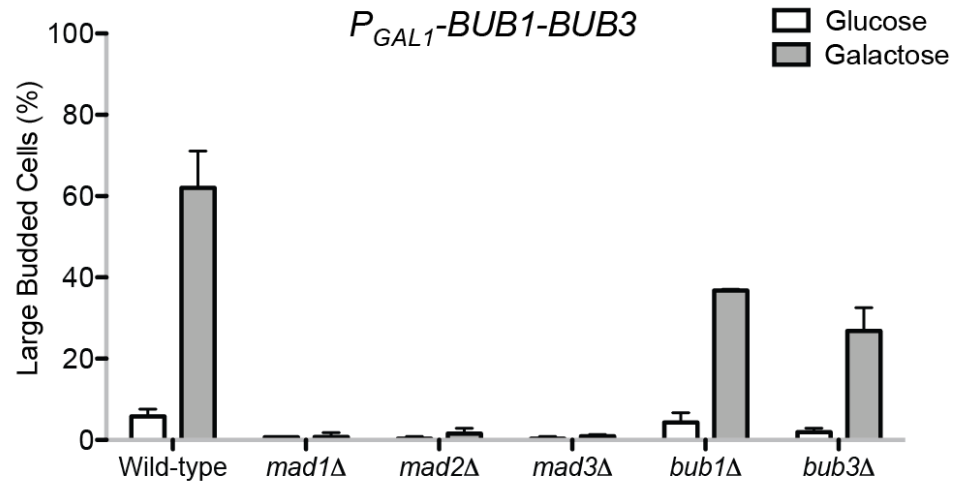
Bub1 had minimal effect on cell cycle progression (**Figure A1-1**). The metaphase arrest induced by Bub1-Bub3 may thus depend on how the two checkpoint proteins are fused together. One possibility is that the specific orientations of Bub1 and Bub3 in Bub1-Bub3 are absolutely required for the downstream effects of the protein fusion. Alternatively, Bub3-Bub1 may fail to arrest cells simply because the checkpoint function of Bub3 is compromised when its C-terminus is fused to another protein, a proposal that could be investigated by testing for the biological activity of a Bub3-GFP fusion.

We then asked whether the two protein fusions depend on other checkpoint components to induce metaphase arrest. We introduced the *P<sub>GALI</sub>-BUB1-BUB3* or *P<sub>GALI</sub>-BUB1-MAD2* construct into yeast strains with different spindle checkpoint genes deleted (*mad1Δ*, *mad2Δ*, *mad3Δ*, *bub1Δ*, and *bub3Δ*) and again tested the effect of expressing the protein fusions. For both Bub1-Bub3 and Bub1-Mad2, cells failed to activate the checkpoint if they had deletion in the checkpoint gene that was not present in the expressed protein fusion (**Figure A1-2A and B**). The results show that both fusions require other spindle checkpoint protein to arrest cells in metaphase.

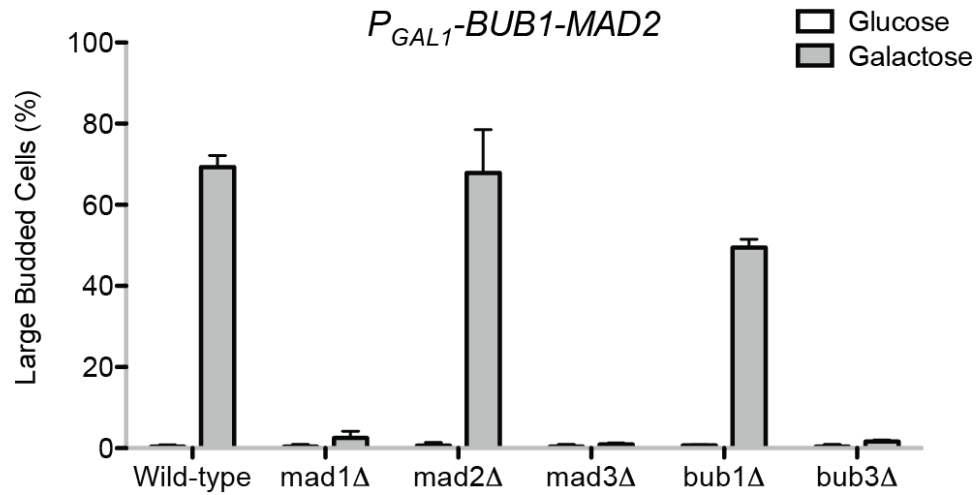
## Conclusions and Discussion

We showed that expressing two protein fusions, Bub1-Bub3 and Bub1-Mad2, is sufficient to induce metaphase arrest in cells. Both fusions are able to activate the checkpoint only in the presence of other checkpoint components, suggesting that they contribute to events upstream in the checkpoint pathway that ultimately lead to inhibition of APC activity.

**A**



**B**



**Figure A1-2.** Checkpoint activation by Bub1-Bub3 and Bub1-Mad2 require most checkpoint components. Cells with **(A)**  $P_{GAL1}$ -*BUB1-BUB3* or **(B)**  $P_{GAL1}$ -*BUB1-MAD2* and deletion of the indicated checkpoint genes were released from G<sub>1</sub> arrest into glucose- or galactose-containing media. The percentage of large budded cells was determined by light microscopy after 3 hours of growth. Error bars represent the standard deviation of three independent trials. Two hundred cells were counted for each trial.

How do these fusion proteins induce metaphase arrest in cells? One explanation is that similar to the Mad2-Mad3 fusion, Bub1-Bub3 and Bub1-Mad2 are able to bind to certain downstream checkpoint targets tightly, and the binding is sufficient to lead to cell cycle arrest. Given the role of Bub1 in bi-orienting chromosomes [1-3], the fusions may also sequester Bub1 targets away from the kinetochore and therefore affect chromosome bi-orientation, leading to mis-aligned chromosomes that in turn activates the checkpoint. Alternatively, the binding of Bub1 to Bub3 or Mad2 may be important for initiating downstream events in the spindle checkpoint and is normally carefully controlled. Expression of Bub1-Bub3 and Bub1-Mad2 fusions force Bub1 to constitutively interact with Bub3 or Mad2 and may thus lead to ectopic checkpoint activation. Future experiments are needed to further characterize the behavior of these protein fusions as well as to test possible models that explain how they arrest cells in metaphase. The results can potentially improve our understanding of the protein interactions that are important for spindle checkpoint activation.

## Materials and Methods

### Yeast Strains and Methods

All strains are derivatives of W303 (*ade2-1 his3-11,15 leu2-3,112 trp1-1 ura3-1 can1-100*), and are listed in **Table A1-1**. Strains were constructed using standard genetic techniques. All media were prepared using established recipes [4], and contain 2% wt/vol of the indicated sugar as the carbon source.

### Cell Cycle Analysis by Light Microscopy

To look at the effect of overexpressing different checkpoint constructs on cell cycle progression, cells were first grown to mid-log phase ( $10^7$  cells/ml) in YEP with 2% raffinose (wt/vol). Cells were then arrested in G<sub>1</sub> by adding 10 $\mu$ g/ml  $\alpha$ -factor (Bio-Synthesis, Lewisville, TX) and incubated for 2 hours at 30°C. Cells were washed four times to remove  $\alpha$ -factor and resuspended in YEP with either 2% glucose (wt/vol) or 2% galactose (wt/vol). After growing for 3 hours at 30°C, the cultures were briefly sonicated to separate cells that fail to dissociate completely after division and the percentage of large-budded cells in each sample was determined by light microscopy.

Table A1-1. Strains used in this Chapter.

Strain Name	Genotype
DLY559	<i>MATa P<sub>GALI</sub>-BUB1-BUB3@URA3</i>
DLY596	<i>MATa P<sub>GALI</sub>-BUB3-BUB1@URA3</i>
DLY599	<i>MATa P<sub>GALI</sub>-MAD2-BUB1@URA3</i>
DLY600	<i>MATa P<sub>GALI</sub>-BUB1-MAD2@URA3</i>
DLY621	<i>MATa P<sub>GALI</sub>-BUB1-BUB3@URA3 mad2Δ::Kan<sup>r</sup></i>
DLY677	<i>MATa P<sub>GALI</sub>-BUB1-BUB3@URA3 bub1Δ::Kan<sup>r</sup></i>
DLY693	<i>MATa P<sub>GALI</sub>-BUB1-BUB3@URA3 mad1Δ::HIS3</i>
DLY694	<i>MATa P<sub>GALI</sub>-BUB1-BUB3@URA3 mad3Δ::Kan<sup>r</sup></i>
DLY709	<i>MATa P<sub>GALI</sub>-BUB1-MAD2@URA3 mad1Δ::HIS3</i>
DLY710	<i>MATa P<sub>GALI</sub>-BUB1-MAD2@URA3 mad2Δ::Kan<sup>r</sup></i>
DLY711	<i>MATa P<sub>GALI</sub>-BUB1-MAD2@URA3 mad3Δ::Kan<sup>r</sup></i>
DLY712	<i>MATa P<sub>GALI</sub>-BUB1-MAD2@URA3 bub1Δ::Kan<sup>r</sup></i>
DLY713	<i>MATa P<sub>GALI</sub>-BUB1-MAD2@URA3 bub3Δ::Kan<sup>r</sup></i>
DLY1198	<i>MATa P<sub>GALI</sub>-BUB1-BUB3@URA3 bub3Δ::Kan<sup>r</sup></i>

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## **Appendix Two**

### **The Interaction Between the Mad2-Mad3 Fusion and Cdc20**

#### **Abstract**

In Chapter Four we described the observation that a Mad2-Mad3 protein fusion induces cell cycle arrest in budding yeast cells when it is overexpressed. The arrest is likely due to the ability of the fusion to bind and inhibit Cdc20. Supporting this model, we found that the Mad2-Mad3 fusion can associate with Cdc20 in vivo. We also showed that while Cdc20 is rapidly degraded when the spindle checkpoint is activated by microtubule poisons, the protein level of Cdc20 is stabilized during metaphase arrest induced by Mad2-Mad3 expression. Our results may reflect the roles of Mad2 and Mad3 in inhibiting the activity of APC<sup>Cdc20</sup>.

## Introduction

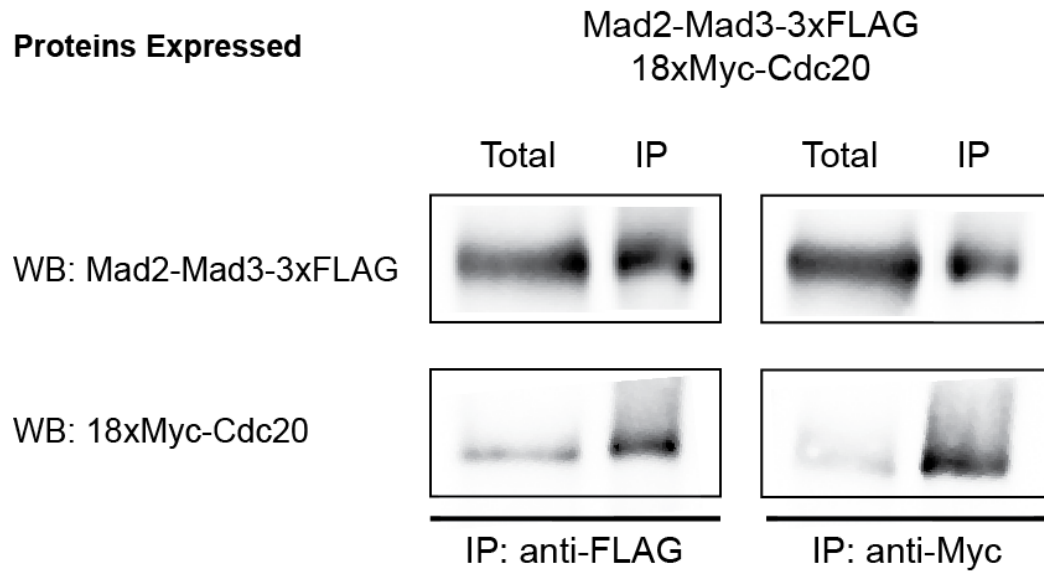
Expressing a Mad2-Mad3 protein fusion can arrest cells in metaphase independently of other checkpoint components (discussed in Chapter Four). We speculate that the Mad2-Mad3 fusion can bind tightly to Cdc20, the main target of the spindle checkpoint, and the binding is sufficient to lead to the inhibition of Cdc20 and APC.

To test the model that Mad2-Mad3 inhibits APC activity by binding to Cdc20, we looked at the interaction between the Mad2-Mad3 fusion and Cdc20. We showed that Mad2-Mad3 can in fact associate with Cdc20 in cells. In addition, we found that the protein level of Cdc20 remains relatively stable in cells that are arrested in mitosis by Mad2-Mad3 expression, which can potentially be explained by the effects of Mad2 and Mad3 on Cdc20 function.

## Results

### The Mad2-Mad3 Fusion Associates with Cdc20

Both Mad2 and Mad3 associate with Cdc20 in budding yeast throughout the cell cycle [1, 2]. We performed coimmunoprecipitation (co-IP) experiments to test whether the Mad2-Mad3 fusion can also bind Cdc20. We introduced *P<sub>GALI</sub>-MAD2-MAD3-3×FLAG* into a strain that expresses epitope-tagged Cdc20 (18×Myc-Cdc20). We released the cells from G<sub>1</sub> arrest into medium with galactose (to express Mad2-Mad3-3×FLAG) for three hours, and prepared lysates from the cultures for co-IP. An anti-FLAG immunoprecipitate contained both 18×Myc-Cdc20 and Mad2-Mad3-3×FLAG, showing that the Mad2-Mad3 fusion can bind Cdc20 (**Figure A2-1**). Similarly, both

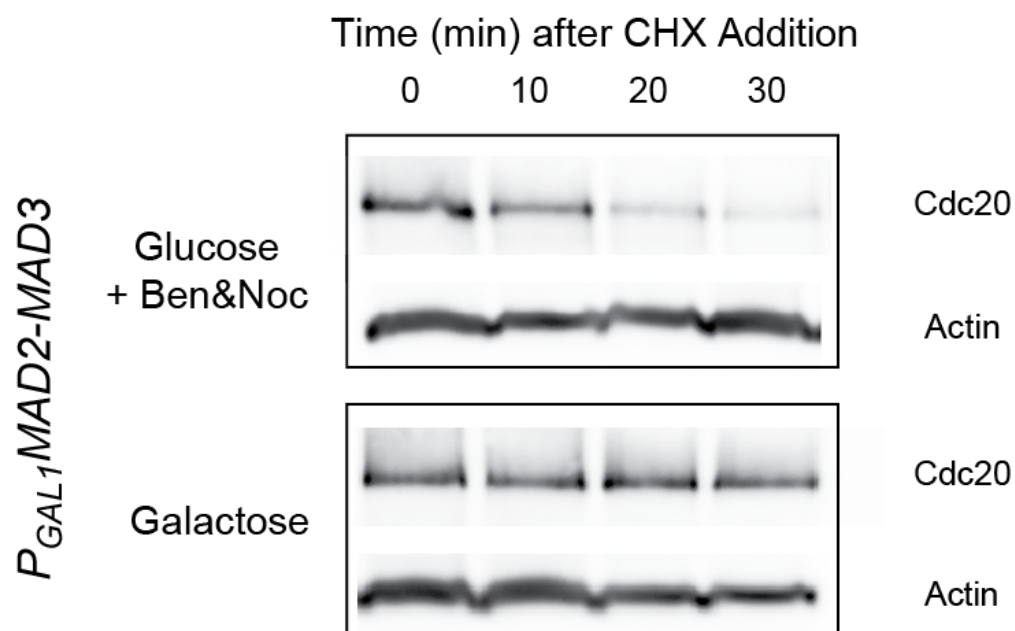


**Figure A2-1.** The Mad2-Mad3 fusion associates with Cdc20. Cells carrying  $P_{GAL1}$ -*MAD2-MAD3-3×FLAG* and  $P_{CDC20}$ -*18×Myc-CDC20* were grown to mid-log phase and arrested in G<sub>1</sub> with  $\alpha$ -factor, and were released into medium with galactose to express Mad2-Mad3-3×FLAG. Extracts were made from the cultures and either Mad2-Mad3-3×FLAG (IP: anti-FLAG) or 18×Myc-Cdc20 (IP: anti-Myc) was immunoprecipitated. The immunoprecipitates (IP) and the total lysates (Total) were then used for Western blots (WB) with anti-FLAG (to visualize FLAG tagged Mad2-Mad3) and anti-Myc (to visualize Myc tagged Cdc20) antibodies (n =3). Both Cdc20 and Mad2-Mad3 were found in the immunoprecipitates, showing that Mad2-Mad3 can bind Cdc20.

epitope-tagged Cdc20 and Mad2-Mad3 can be found in an anti-Myc immunoprecipitate, confirming that the two proteins can interact (**Figure A2-1**).

### **Cdc20 is Stabilized in Cells Arrested in Metaphase by Mad2-Mad3 Expression**

The Mad2-Mad3 fusion can potentially inhibit the activity of APC<sup>Cdc20</sup> through multiple mechanisms. The protein fusion may prevent APC<sup>Cdc20</sup> from recognizing its substrates. Mad2-Mad3 may also stimulate the degradation of Cdc20 and thus interferes with the activation of APC activity. To look at how expression of Mad2-Mad3 affects Cdc20, we monitored the level of epitope tagged Cdc20 (18×Myc-Cdc20) during cell cycle arrest. Cells carrying *P<sub>GALI</sub>-MAD2-MAD3* were released from G<sub>1</sub> arrest into either glucose-containing medium (to inhibit expression of Mad2-Mad3) with benomyl and nocodazole (to depolymerize microtubule) or galactose-containing medium (to express Mad2-Mad3). After all the cells were arrested in metaphase, cycloheximide was added to the media to inhibit protein synthesis, and the protein level of Cdc20 was determined by Western blots. When the cells were arrested in metaphase by microtubule poisons, the level of Cdc20 decreased rapidly over time (**Figure A2-2**). Most of the Cdc20 disappeared by 30 minutes after the addition of cycloheximide. The result agrees with previous reports showing that Cdc20 is degraded during checkpoint activation [3, 4]. In contrast, Cdc20 was stabilized in cells arrested by expression of the Mad2-Mad3 fusion (**Figure A2-2**). In the presence of both the Mad2-Mad3 fusion and microtubule poisons, the protein level of Cdc20 also remained stable (data not shown). Mad2-Mad3 thus prevents the degradation of Cdc20 that is normally observed when the spindle checkpoint is activated.



**Figure A2-2.** Expression of the Mad2-Mad3 fusion stabilizes Cdc20. The protein level of Cdc20 was monitored by Western blots (n=3). Cells with Mad2-Mad3 under the *GAL1* promoter and 18×Myc-Cdc20 were grown to mid-log phase and arrested in G<sub>1</sub> with  $\alpha$ -factor, and were released into media with either glucose plus benomyl and nocodazole (top) or galactose (bottom). After two hours, cycloheximide (CHX) was added to the cultures to inhibit protein synthesis. Western blots against Myc (to visualize Myc-tagged Cdc20) or actin (loading control) were then performed. Cdc20 was rapidly degraded when cells are arrested in metaphase by microtubule poisons. On the other hand, the protein level of Cdc20 stayed relatively constant when Mad2-Mad3 was overexpressed, indicating that the Mad2-Mad3 fusion stabilizes Cdc20.

## Conclusions and Discussion

It is not clear how expression of the Mad2-Mad3 fusion is sufficient to arrest cells in metaphase. One model is that the fusion can associate with Cdc20 tightly, and this binding ultimately leads to the inhibition of APC activity and cell cycle arrest. We showed that Mad2-Mad3 fusion is able to bind Cdc20. Moreover, the protein level of Cdc20 stays relatively constant when cells are arrested in mitosis by the Mad2-Mad3 fusion, indicating that the binding of Mad2-Mad3 to Cdc20 has a strong effect on its stability.

Both Mad2 and Mad3 can interact with Cdc20 [1, 2], therefore it is not surprising that the Mad2-Mad3 fusion can also bind Cdc20. While the co-IP results agree with the hypothesis that Mad2-Mad3 inhibits APC activity by binding to Cdc20 tightly, we have to characterize the nature of this interaction to further support the model. One future direction is to perform in vitro biochemical assays using purified Mad2-Mad3 fusion and Cdc20 to determine whether the properties of the binding between these proteins are significantly different from that of the association between Cdc20 and Mad2 or Mad3 alone.

We showed that when the spindle checkpoint is activated by microtubule poisons, Cdc20 is rapidly degraded, confirming previous reports that Cdc20 is unstable during checkpoint activation [3, 4]. On the other hand, when cells are arrested in mitosis by the expression of Mad2-Mad3, the protein level of Cdc20 is stabilized. One explanation for the difference in Cdc20 stability is that Mad2 and Mad3 inhibit APC activity through different mechanisms. Mad2 binds to the N-terminus of Cdc20 and affect the interaction between Cdc20 and APC, which is important for the activation of APC [5, 6]. Mad3 is

important for preventing Cdc20 from binding its substrates, mainly by occupying the KEN box-binding site of Cdc20 [6-9]. During spindle checkpoint activation, Cdc20 interacts with both Mad2 and Mad3, and these interactions are very dynamic. In cases when Cdc20 is bound by Mad3 but not Mad2, Cdc20 can still interact with APC through the C box, and APC will subsequently target Cdc20 for degradation. Cdc20 is thus degraded during checkpoint activation. In contrast, in the presence of the Mad2-Mad3 fusion, Cdc20 always associates with both Mad2 and Mad3 at the same time since Mad2 and Mad3 are linked covalently. The binding of Mad2 prevents the interaction between Cdc20 and APC, and Cdc20 is therefore stabilized in cells arrested in metaphase by Mad2-Mad3. One possible experiment to test this idea is to determine the stability of Cdc20 when it is tethered to either Mad2 or Mad3 using engineered leucine zippers. The results may ultimately explain how the Mad2-Mad3 fusion is able to inhibit the activity of APC<sup>Cdc20</sup>.

## Materials and Methods

### Yeast Strains and Methods

All strains are derivatives of W303 (*ade2-1 his3-11,15 leu2-3,112 trp1-1 ura3-1 can1-100*), and are listed in **Table A2-1**. Strains were constructed using standard genetic techniques. All media were prepared using established recipes [10], and contain 2% wt/vol of the indicated sugar as the carbon source. To prepare media containing benomyl and nocodazole, DMSO stocks of methyl 1-(butylcarbamoyl)-2-benzimidazolecarbamate (benomyl) (Sigma-Aldrich, St. Louis, MO) and nocodazole (Sigma-Aldrich, St. Louis MO) were added to yeast extract and peptone (YEP) with 2% wt/vol glucose to a final concentration of 30µg/ml of each drug.

### Co-Immunoprecipitation

For immunoprecipitation experiments, cells were grown to mid-log phase ( $10^7$  cells/ml) in 20 ml of YEP with 2% raffinose (wt/vol). Cells were then arrested in G<sub>1</sub> by adding 2µg/ml  $\alpha$ -factor (Bio-Synthesis, Lewisville, TX) and incubated for 2 hours at 30°C. Cells were washed four times to remove  $\alpha$ -factor and resuspended in YEP with 2% galactose (wt/vol). After three hours of growth, cells were harvested by brief centrifugation and the pellets were immediately frozen with liquid nitrogen. Yeast extracts were made by vortexing frozen cell pellets in 200µl of lysis buffer (100mM NaCl, 50mM Tris-HCl pH7.5, 10% glycerol, 50mM NaF, 50mM Na- $\beta$ -glycerolphosphate, 2mM EDTA, 0.1% Triton X-100, 1mM Na<sub>3</sub>VO<sub>4</sub>, 1mM PMSF, protease inhibitor cocktail) and around 100µl of acid washed glass beads for three rounds of one minute, incubating on ice for one minute between each round. The resulting lysate was separated



from the glass beads and centrifuged at 14,000 rpm for five minutes at 4°C to remove insoluble material. A portion of the lysate was mixed with an equal volume of 2X SDS sample buffer (Total).

Antibody was added to the remaining lysate at 1:50 dilution and incubated on ice for one hour. Samples were then transferred to 30µl of Protein A/G PLUS-agarose beads (Santa Cruz Biotechnology, Santa Cruz, CA) which had been equilibrated in lysis buffer. The beads were rotated at 4°C for one hour. The beads were then washed four times with lysis buffer and boiled in SDS sample buffer for ten minutes. The resulting samples (IP) were used for Western blotting.

### **Cell Cycle Experiment to Look at the Stability of Cdc20**

To look at the stability of Cdc20 during metaphase arrest, cells were first grown to mid-log phase ( $10^7$  cells/ml) in YEP with 2% raffinose (wt/vol). Cells were then arrested in G<sub>1</sub> by adding 2µg/ml  $\alpha$ -factor (Bio-Synthesis, Lewisville, TX) and incubated for 2 hours at 30°C. Cells were washed four times to remove  $\alpha$ -factor and resuspended in YEP with either 2% glucose (wt/vol) with benomyl and nocodazole or 2% galactose (wt/vol). After growing for 2 hours at 30°C, protein production was shut off by adding 1 mg/mL cycloheximide (Sigma-Aldrich, St. Louis MO). At the indicated time points, 1ml samples of the culture were collected. Cells were pelleted by centrifugation for 1 min at room temperature. The supernatant was removed, and cell pellets were stored at -80°C. Cell pellets were lysed using a NaOH/ $\beta$ -mercaptoethanol-based protocol [11] and the resulting lysates were used for Western blots.

## **Western Blots**

Proteins samples were loaded onto and separated in 10% Criterion Tris-HCl polyacrylamide gel (Bio-Rad, Hercules, CA). Proteins were transferred overnight to nitrocellulose (Whatman, Picataway, NJ). Western blotting for Myc-tagged Cdc20 were performed using anti-Myc 9E10 antibodies (Roche Applied Science, Indianapolis, IN) at a 1:500 dilution, FLAG-tagged Mad2-Mad3 was detected with anti-FLAG antibodies (Sigma-Aldrich, St. Louis, MO) used at a 1:2000 dilution, and actin was detected with anti-actin antibodies (Abcam, Cambridge, MA) used at a 1:2000 dilution. Horseradish peroxidase-conjugated goat anti-mouse (Santa Cruz Biotechnology, Santa Cruz, CA) was used as the secondary antibody at a 1:2000 dilution. The secondary antibody was detected by SuperSignal West Dura Chemiluminescent Substrate (Thermo Fisher Scientific, Rockford, IL) and the blot was imaged with an AlphaImager (ProteinSimple, Santa Clara, CA).

Table A2-1. Strains used in this Chapter.

Strain Name	Genotype
DLY1081	<i>MATa 18×MYC-CDC20 @TRP1 P<sub>GALI</sub>-MAD2-MAD3-3×FLAG@URA3 ssd1</i>
DLY1139	<i>MATa 18×MYC-CDC20 @TRP1 P<sub>GALI</sub>-MAD2-MAD3@URA3 ssd1</i>

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